Renal cell carcinoma : risk factors and von Hippel-Lindau gene mutations

Renal Cell Carcinoma

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Voor pap en mam

Contents

1

Introduction

Kidney cancer includes cancer of the renal parenchyma (Renal Cell Carcinoma (RCC)) and cancer of the renal pelvis. These two different types of cancer not only differ with respect to anatomical location and the type of cell from which these evolve (parenchymal cells versus urothelial cells, respectively), but also with respect to their association with risk factors. Unfortunately, the distinction is not always made, as is the case for most descriptive rates of kidney cancer.

Descriptive epidemiology

Kidney cancer (including cancer of the renal pelvis) is the ninth most common cancer in the European Union[1]. Incidence and mortality rates for 2002 are shown in Table 1.1. These are approximately twice as high for men as for women[1,2].

Table 1.1 Age standardized (world) incidence and mortality rates (ASR(W)) per 100,000 person years for kidney cancer - 2002.

Country/region	Men		Women	
	Incidence (ASR(W))	Mortality (ASR(W))	Incidence (ASR(W))	Mortality (ASR (W))
World	4.7	2.3	2.5	1.2
More developed regions	10.4	4.6	5.0	2.1
Less developed regions	2.1	1.2	1.2	0.7
Northern America	12.6	4.1	5.9	2.0
Western Europe	11.3	5.2	5.4	2.5
The Netherlands	9.8	5.5	4.9	2.7

Source: http://www-dep.iarc.fr/[2]

Also, incidence and mortality rates are clearly higher for more developed regions, including The Netherlands, than for less developed regions[2]. In some parts of the world an increase in incidence of kidney cancer has been reported[3-6], but in The Netherlands the incidence of kidney cancer (excluding cancer of the renal pelvis) remained stable[7]. This reported increase in incidence may partly be due to an increase in abdominal imaging use[3-6], resulting in a larger number of incidentally discovered RCC. If this were the case, one would expect this increase to occur specifically in small, localized tumors. In a report by Hock *et al.*, an increase in the percentage of localized tumors was observed in the period from 1986 through 1998 compared to the period of 1973 through 1985, but this was not statistically significant[3]. In that study, the annual percent change in incidence rate was also considered and this rate increased in localized, regionally advanced and distant tumors with the highest increase in the localized group[3]. Trends in mortality rates show a non-significant decrease[4], but in the Netherlands no change in mortality rate for kidney cancer, excluding cancer of the renal pelvis, was observed[7]. The possible decrease in mortality rates may be the result

of an increase in 5-year survival. European estimates for 5-year survival have increased from approximately 40%[8] for patients diagnosed in 1978-1980 to approximately 55% for patients diagnosed in 1990-1994[9]. Generally, this may reflect better treatment or the treatment being more effective because the cancer was diagnosed at an earlier stage[8].

Pathology and genetics

We confined our research to cancer of the renal parenchyma, RCC, which can be subdivided into several histological types. The most common histological subtype is clear-cell or conventional RCC, which accounts for approximately 75% of renal cell neoplasms in surgical series[10]. This tumor is characterized by clear cytoplasm, hence the name clear-cell RCC[10]. The other subtypes are papillary RCC, chromophobe RCC, collecting duct carcinoma and unclassified RCC.

Specific genetic alterations have been linked to each of these histological subtypes. Aberrations in the *von Hippel-Lindau (VHL)* gene have been shown to be an early and distinct event in the development of clear-cell RCC[11]. The *VHL* gene was identified after molecular research of DNA from patients with Von Hippel-Lindau disease, a hereditary syndrome that predisposes to, amongst others, clear-cell RCC. Allelic imbalance was frequently observed at 3p25-26[12-14]. The corresponding gene was characterized as a tumor suppressor gene and named the *von Hippel-Lindau* gene[14,15]. The *VHL* gene is composed of 3 exons.

VHL gene mutations have been observed in 56-69% of sporadic (non-hereditary) clear-cell RCCs[14]. Mutations usually lead to a shortened inactive protein. The VHL protein may also be absent as a result of hypermethylation of the promotor area. This is estimated to occur in approximately 15-19% of sporadic clear-cell RCCs[14,16]. Since the *VHL* gene is a tumor suppressor gene, both alleles need to be affected before it loses its function. Loss of heterozygosity (LOH) is common and inactivates one allele. LOH has been reported to occur in 74%[17] to 93%[18] of tumors. Kondo *et al.* showed 97% LOH in mutated or hypermethylated samples compared to 81% LOH in wildtype samples[19].

The non-mutated human *VHL* gene is translated into two wildtype VHL proteins: pVHL30 and pVHL19[20,21]. Both proteins are capable of suppressing tumor formation[21]. Under normal oxygen conditions, pVHL forms a complex with elongin B, elongin C and cullin 2 proteins[14,20], which promotes ubiquitin-mediated proteasomal degradation of hypoxia inducible factor (HIF). Loss of pVHL leads to an active HIF complex, which binds to the hypoxia-responsive elements within the promoter/enhancer of hypoxia inducible genes, thus activating hypoxia-inducible genes[14,20], even under normal oxygen conditions. The result is angiogenesis (blood vessel formation), which is necessary for solid tumors to grow larger than 1-2 mm^3 [13]. In addition, VHL expression can restore the ability of RCC cell lines to exit the cell cycle and enter G0 quiescence upon serum withdrawal in culture[22]. pVHL30 has an additional role in the assembly of extracellular fibronectin matrix[20,21]. Loss of pVHL function, i.e. loss of

regulation of hypoxia inducible factors, no promotion of "cell cycle exit", and no promotion of correct formation of the fibronectin extracellular matrix[14], creates an environment favorable for tumor growth. A clear summary of possible interactions and functions of the wildtype VHL protein is given in a review by Richards[14].

Risk factors

Many risk factors have been investigated in association with RCC risk. Some risk factors, such as cigarette smoking[23-26], obesity[23-27] and a history of hypertension and/or use of anti-hypertensive medication[23,25], were consistently reported to be positively associated with RCC risk, although rate ratios (RRs) were only moderately increased. Reports on other risk factors showed less consistent associations with RCC risk. A literature overview of risk factors, for which their association with RCC was investigated, is given in Table 1.2.

In the following sections, some details for risk factors that are consistently linked to RCC risk are discussed.

A recent meta-analysis of 19 case-control studies and 5 cohort studies on cigarette smoking in relation to RCC concluded that inhaled tobacco smoke is clearly implicated in the etiology of RCC. Risk was increased for former (RR: 1.21; 95% confidence interval (CI): 1.07-1.36) and current (RR: 1.45; 95% CI: 1.26-1.66) smokers with a strong dose-dependent increase in risk[28]. Population-based attributable risks indicate that approximately 20% to 30% of RCC among men and 10% to 20% among women can be accounted for by cigarette smoking[23,24]. Also, it was shown that cigarette smoke metabolites can cause mutations in human DNA, which is not restricted to tissues directly exposed to tobacco smoke[29]. Cigarette smoke metabolites were found in the urine of smokers[30], implying exposure of the kidney to mutagenic cigarette smoke metabolites.

Body mass index (BMI), the ratio of weight (kg) to the square of height (m^2), is believed to be an important risk factor for RCC. In a meta-analysis of 4 cohort studies, 8 population-based case control studies and 3 hospital-based case control studies, a summary RR of 1.07 (95% CI: 1.05-1.09) per 1 kg/m^2 increase in BMI was found for men and women together[31]. The attributable proportion of RCC for excess body weight is estimated to be 25% for both men and women in the European Union[32].

Hypertension and use of diuretics or other antihypertensive medication appeared to be risk factors for RCC in many epidemiological studies[33,34]. In a meta-analysis, a pooled adjusted odds ratio (OR) of 1.75 (95% CI: 1.61-1.90) was calculated for the association of hypertension, defined as a systolic blood pressure greater than or equal to 160 mm Hg, and RCC[33]. This pooled OR did not include prospective cohort studies, which also found increased risks of RCC with increasing blood pressure[35-38]. For diuretic use, a pooled OR of 1.55 (95% CI: 1.42-1.77) was found based on case-control studies only[34]. Three cohort studies were also suggestive of an association between diuretic use and

RCC[39-41]. However, it is unclear whether hypertension and antihypertensive medication, specifically diuretics, or just one of these factors is responsible for the increased risk. Some recent studies showed that diuretic medication was no longer a risk factor after controlling adequately for the diagnosis of hypertension[42,43], which suggests that not medication but hypertension itself is a risk factor for RCC.

Table 1.2 Literature overview of risk factors for renal cell carcinoma (RCC)[a].

Factors consistently linked to RCC risk	Direction of association[b]
Cigarette smoking	+
Obesity	+
History of hypertension	+
Diuretic use	+
Factors inconsistently linked to RCC risk	**Direction of association[b]**
Vegetable and fruit consumption	-
Micronutrient and vitamin intake	-
Dairy product consumption	+
Protein intake	+
Fat intake	+
Meat consumption	+
Alcohol consumption	-
Coffee drinking	+/-
Tea drinking	+/-
Physical activity	-
Occupational exposure to asbestos	+
Occupational exposure to polycyclic aromatic hydrocarbons	+
Occupational exposure to gasoline and other petroleum products	+
Occupational exposure to solvents, such as trichloroethylene	+
Exposure to radiation	+
Exposure to heavy metals (e.g., cadmium)	+
Oral contraceptive use	-
Estrogen replacement therapy	+
Increasing parity	+
Age at menarche	-
Age at first birth	-
History of hysterectomy / oophorectomy	+
Analgesic use	+
Acquired cystic disease	+
Kidney stones	+
Urinary tract infection	+
Diabetes	+
Low socioeconomic status	+

[a] This table is based on several reviews[23-27]; [b] + Positive association, - Negative association.

In the following sections, some details for risk factors inconsistently reported to be associated to RCC risk are given.

Dietary factors have also been investigated in association with RCC; positive associations were reported for dairy products, intake of protein and fat, and meat while inverse associations were reported for vegetable and fruit consumption, intake of dietary antioxidants, and alcohol consumption[23-26].
RCC is not generally thought of as an occupationally induced tumor, but the associations of a large number of occupational factors to RCC have been investigated. Workers exposed to degreasing agents and solvents (e.g., trichloroethylene) have been reported to be at increased risk for RCC, but the results are inconsistent[23-26]. Also, exposure to some specific occupational risk factors, such as asbestos, gasoline and other petroleum products, hydrocarbons, lead, and cadmium have been associated to an increased RCC risk[23-26].
There have also been some reports on the association of reproductive factors to RCC, but no convincing consistent results have been observed[24,25].

Rationale for this study

As has been shown in the previous section, associations between risk factors and RCC risk remain weak or inconsistent. Mechanisms linking risk factors to DNA damage, which may ultimately lead to a tumor, have been described for several risk factors. However, the endpoint RCC, which is commonly used, is heterogeneous in that RCCs differ in histology and in the presence of DNA mutations. Therefore, stratified analyses based on histology and the mutational status of a tumor may lead to additional insight in the carcinogenesis process. In that respect, clear-cell RCC and the presence of *VHL* gene mutations are a suitable endpoint to study in association to risk factors, since clear-cell RCC is the most common histological subtype and mutations in the *VHL* gene are an early event in clear-cell renal cell carcinogenesis. This is shown by the high frequency of observed *VHL* gene mutations in clear-cell RCC and the presence of these mutations in all stages. If heterogeneous results are observed, these may serve as a clue for fundamental etiological processes in the carcinogenesis of clear-cell RCC.
Thus far, only two studies have linked risk factors to the presence of *VHL* gene mutations. The first was a case-control study by the group of Brauch *et al.*[44,45], the other was a case-only study by Hemminki *et al.*[46].
The group of Brauch *et al.* conducted a case-control study to investigate whether trichloroethylene exposure produced RCC through a specific mutational effect on the *VHL* gene[44]. Thirty-three of 44 (75%) RCC cases with occupational exposure to trichloroethylene presented with at least one *VHL* gene mutation, in comparison to 42 out of 73 (58%) of the RCC cases not occupationally exposed to trichloroethylene. In the group of cases occupationally exposed to trichloroethylene, a specific mutation was observed in 13/33 (39%) of mutations. This C>T missense mutation at nucleotide 454, changing a proline to a serine within the VHL protein at codon 81, was not observed in lymphocyte DNA from these patients or from 97 control individuals without RCC. This

mutation was also not present in tumor DNA from 107 RCC cases, not occupationally exposed to trichloroethylene, indicating specificity[44]. In an additional study on partly the same patients and a non-exposed control group, no differences in tumor type, grade and stage were observed, but trichloroethylene exposed patients were diagnosed at a statistically significantly younger age than non-exposed patients. *VHL* gene mutations were observed in 14/17 (82%) tumors of exposed RCC cases, compared to 2/21 (10%) tumors of the non-exposed group RCC cases, but *VHL* gene mutation analyses were hampered by technical problems related to the quality of archival DNA[45].
Hemminki *et al.* obtained detailed exposure information through a personal interview using a structured questionnaire and DNA from archival paraffin-embedded material for 102 male patients with RCC. Forty-seven of 102 (46%) patients presented with at least one mutation. Consumption of vegetables was associated with a decreased frequency of *VHL* gene mutations among smokers, while citrus fruit consumption was associated with a decreased frequency of *VHL* gene mutations among all patients. The authors concluded that these results provide evidence that vegetable and citrus fruit consumption protect the renal *VHL* gene from mutational insults, although chance findings could not be ruled out[46]. Additionally, it has to be noted that all investigated persons were diagnosed with RCC, so no comparison to the level of vegetable and citrus fruit consumption in the general population could be made.

Aim of the study

To investigate the associations of several risk factors and RCC risk, and to further investigate these associations by redefining the endpoint based on histology and *VHL* gene mutational status.

Study design

Data from the Netherlands cohort study on diet and cancer (NLCS), a large nationwide population-based cohort study that started in 1986 in a joined effort by Maastricht University and TNO Nutrition and Food Research[47], was used. In the design phase, an effort was undertaken to reduce loss to follow-up to a minimum, by choosing areas with a good coverage by the National Cancer Registry and PALGA, a nationwide pathology database[48]. Linkage to the PALGA database also gave us the unique opportunity to use paraffin embedded tumor material, since the location of paraffin material was recorded in this database. Paraffin material from 51 pathology laboratories was used to review histology and investigate the mutational status of RCCs, which enabled us to further define the endpoint for analyses.

The study design of The Netherlands cohort study, including collection of paraffin material and statistical analyses, as carried out in the present study, is shown in Figure 1.1.

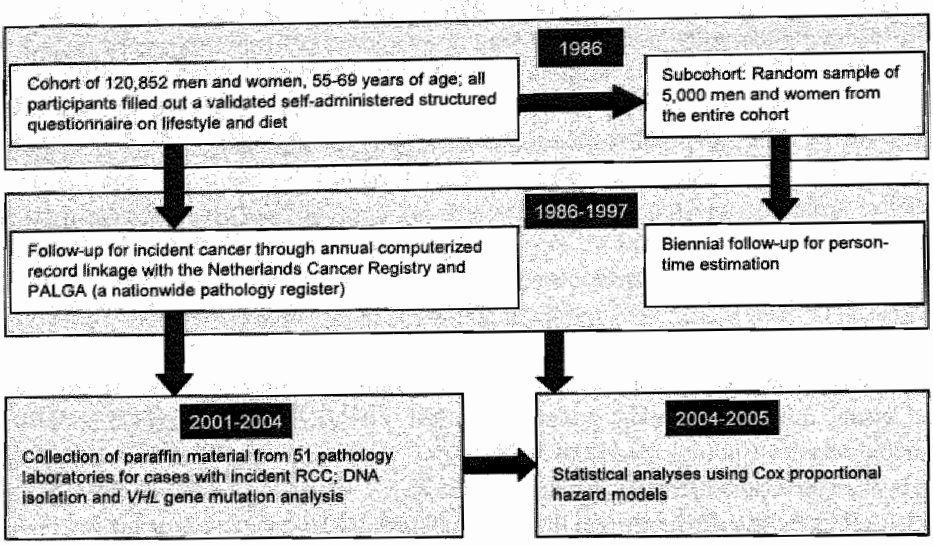

Figure 1.1 Study design – Netherlands cohort study on diet and cancer.

A case-cohort approach was used, which means that cases were enumerated for the entire cohort, while the person-time accumulated in the entire cohort was estimated using a subcohort of 5,000 men and women, which was randomly drawn from the entire cohort at baseline. After 11.3 years of follow-up 337 incident cases were observed. In this thesis, we focus on clear-cell RCC because this is the most common subtype of RCC, and will therefore generate a sufficiently large number of cases to investigate associations with risk factors. Additionally, aberrations in the *VHL* gene are an early and distinct event in the development of clear-cell RCC.

Outline of this thesis

In the next two chapters (chapters 2 and 3) associations between risk factors and RCC risk are described. The association between anthropometric variables and RCC risk is explored in chapter 2, which additionally includes information on energy intake and physical activity. In chapter 3, the association of vegetable and fruit consumption and RCC risk is described. Chapter 4 describes the collection of paraffin material, methods and results of *VHL* gene mutations analysis, and the association of mutations with clinical/pathological parameters. In chapters 5, 6, and 7 the relation of cigarette smoking, hypertension and use of antihypertensive medication, and carotenoid and

vitamin intake and RCC risk and *VHL* gene mutations are described, respectively. Finally, in chapter 8, main findings, strengths and limitations, implications of analyses including *VHL* gene mutations, conclusions, and suggestions for further research are discussed.

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2

Relation of height, body mass, energy intake, physical activity to risk of renal cell carcinoma: results from the Netherlands cohort study

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Abstract

Data from the Netherlands Cohort Study on diet and cancer (NLCS) were used to investigate the association between anthropometry, energy intake and physical activity and risk of renal cell carcinoma (RCC). The NLCS consists of 120,852 men and women aged 55-69 years who completed a self-administered questionnaire at baseline (1986). A case-cohort approach was used. After 9.3 years of follow-up, 275 microscopically confirmed incident cases were available for analysis. Incidence rate ratios for RCC were estimated using Cox proportional hazard models. Height was associated with RCC risk only in women (per 5-cm increment, rate ratio (RR): 1.23; 95% confidence interval (CI): 1.03-1.46). Body mass index (weight (kg)/height $(m)^2$) was associated with increased risk of RCC (per 1- kg/m^2 increment, RR: 1.07; 95% CI: 1.02-1.12) for men and women, as was gain in body mass index from age 20 years to baseline (per 1-kg/m^2 increment, RR: 1.06; 95% CI: 1.01-1.10). Energy intake was not related to RCC risk, while a possible protective effect was observed for physical activity in men. These results suggest that body mass index and gain in body mass index since age 20 are associated with increased risk of RCC.

Introduction

Body mass index (BMI), the ratio of weight (kg) to the square of height (m^2), is believed to be an important risk factor for Renal Cell Carcinoma (RCC). In a quantitative review, a summary relative risk per 1-kg/m^2 increase in BMI of 1.07 (95% confidence interval (CI): 1.05-1.09) was found for men and women[1]. The attributable proportion of RCC for excess body weight is estimated to be 25% for both men and women in the European Union[2]. Most studies have reported an association between BMI and RCC for both sexes[3-10], but some studies have reported associations confined to men[11-13] or women[14,15]. Inconsistent reports exist on the association between height and RCC risk[10,13,16]. High body weight is associated with increased risk of RCC, particularly among women[17,18] and weight changes should be investigated further[18]. Reports on energy intake[15,19] and physical activity[16,20,21] have been inconsistent.

BMI is a measure of body mass relative to height. In general, there is good correlation between BMI and percentage of body fat. Obesity or excess body fat is caused by excess energy intake relative to energy expenditure, which consists of resting metabolic rate, the thermic effect of food, posture and spontaneous activity, and voluntary physical activity[22].

Two different biological mechanisms have been proposed to explain the observed relation between BMI and renal cancer risk. Physical activity and energy intake may also fit these mechanisms. Yu and Rohan[23] proposed that the insulin-like growth factor I (IGF-I) system may be the mechanistic link between obesity and the development of RCC. IGF-I has been shown to stimulate cell proliferation and inhibit apoptosis[23], both of which favor tumor growth. In humans, it has been shown that obese persons have increased serum levels of free IGF-I[22]. Overnutrition has been reported to increase levels of IGF-I[23], while no conclusions can be drawn regarding the effect of physical activity on IGF-I levels[22,24-28].

Secondly, the process of lipid peroxidation may be involved[29]. Byproducts of lipid peroxidation have been shown to react with renal cell DNA to form adducts, which may lead to mutations. Obese subjects are known to exhibit increased lipid peroxidation, while exercise programs resulted in reduced lipid peroxidation. Furthermore, it has been proposed that lipid peroxidation may also explain the roles of other risk factors, such as smoking, and protective factors, such as intake of foods high in antioxidants, in the development of RCC[29].

Hence, it would be logical to investigate BMI, energy intake and physical activity simultaneously. To our knowledge, only one study investigated the roles of BMI, physical activity and energy intake together[21], but the emphasis of that study was on physical activity[21]. In this study, a prospective cohort study with a relatively large number of cases, we estimated the effects of height, weight, BMI, BMI at age 20 years, BMI change since age 20 years, energy intake, and physical activity on risk of RCC. We also investigated the effects of BMI, energy intake and physical activity simultaneously.

Materials and methods

The Netherlands Cohort Study on diet and cancer

The study design, including data-collection strategies, has been described in detail previously[30]. Briefly, this population-based prospective cohort study on diet and cancer started in the Netherlands in September 1986. The cohort includes 58,279 men and 62,573 women who were aged 55-69 years at baseline. The study was designed as a case-cohort study, using all cases and a random sample of 5,000 persons from the cohort (subcohort), who have been followed for estimation of the accumulated person-years in the entire cohort[31].

Follow-up

Incident cancer cases occurring in the total cohort were identified through record linkage to the Netherlands cancer registries and PALGA (the national automated pathology archive)[32]. The completeness of cancer follow-up was estimated to be more than 96%[33]. This gave us 275 incident cases (179 men and 96 women) with microscopically confirmed adenocarcinoma of the renal parenchyma and no prevalent cancer at baseline.
Follow-up of the subcohort was almost complete; out of the 5,000 subcohort members (2,411 men and 2,589 women), only two men were lost to follow-up after 9.3 years (September 1986 - December 1995). Subcohort members with prevalent cancer (other than skin cancer) at baseline were excluded (76 men and 145 women) from the analyses, leaving 4,779 subcohort members (2,335 men and 2,444 women).

Questionnaire

At baseline, all cohort members completed a self-administered questionnaire, which has been described elsewhere[34]. Questions were asked about current height, current weight, weight at age 20 years, family history of cancer, physical activity, job history and usual consumption of food and beverages during the year preceding the start of the study.
BMI was calculated by dividing weight (kg) by height squared (m^2). Rate ratios are presented per 1-kg/m^2 increment for BMI at baseline, BMI at age 20 years, and BMI gain between age 20 years and baseline. In addition, BMI at baseline was categorized into the following categories: BMI<23, 23≤BMI<25, 25≤BMI<27, 27≤BMI<30, and BMI≥30. Because of missing values, analyses for BMI were based on 264 incident cases and 4,592 subcohort members. For BMI at age 20 years, participants were categorized into four groups: BMI<20, 20≤BMI<21, 21≤BMI<23, and BMI≥23. Gain in BMI (kg/m^2) was categorized as <0, 0-4, 4-8, or ≥8. Analyses for BMI at age 20 and BMI gain since age 20 were based on 227 cases and 3,905 subcohort members, since not all participants provided information on their weight at age 20.

Energy-intake was calculated from the food frequency questionnaire[34,35] using the computerized Dutch Food Composition table[36]. Fifteen cases and 338 subcohort members with incomplete or inconsistent dietary data were excluded from the analyses. Details are given elsewhere[34]. On the basis of the distribution in the subcohort, energy-intake was divided into quintiles for men and women separately.
Physical activity was divided into occupational and nonoccupational activity. In this paper, we use the term "nonoccupational physical activity" to cover both recreational physical activity and the physical activity involved in getting to and from work (e.g., walking, cycling). For estimation of occupational activity, participants were asked to report job title(s) and job duration(s). Assessment of physical activity at work was based on the job held for the longest amount of time. Total energy expenditure was based on a rating system developed by Hettinger *et al.*[37]. Participants were classified into three energy expenditure groups: <8 kJ/minute, 8-12 kJ/minute, and ≥12 kJ/min. Occupational physical activity was not calculated for women, since most women of this generation had not held a job or had worked for only a short period of time, mostly in the distant past. Baseline nonoccupational physical activity was calculated by adding up the number of minutes spent per day on cycling/walking to work, shopping, and walking the dog, and the number of hours spent per week on gardening/odd jobs, recreational cycling/walking and sports/exercise, as reported[38].

Statistical analysis

On the basis of the literature, age (continuous variable), sex and cigarette smoking (current smoking (yes/no), number of years of smoking, and number of cigarettes smoked per day) were considered as confounders. We did not adjust for family history of RCC (present or not present in a first-degree relative), since only 49 participants (4 cases and 45 subcohort members) reported having a first-degree relative with RCC. Incidence rate ratios for height and weight were obtained from models in which both variables were always entered simultaneously. Results for BMI are additionally adjusted for energy intake and physical activity. We also investigated whether BMI at age 20 (as a proxy for young adulthood) or BMI gain between age 20 and baseline were independent predictors of RCC risk. In all BMI gain regression models, we adjusted for BMI at age 20. Furthermore, we calculated RCC rate ratios for energy intake and physical activity variables (entered into a model together) with adjustment for age only and adjustment for age, smoking and BMI. Results are shown for men and women together to enhance the precision of our statistical analyses, unless the p value for the interaction term between sex and the variable of interest was less than or equal to 0.05.
Rate ratios and 95 percent confidence intervals for RCC were estimated in Cox proportional hazards models using 2001 STATA statistical software (release 7; Stata Corporation, College Station, Texas), after testing the proportional hazards assumption using scaled Schoenfeld residues[39]. Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by

sampling from the cohort[40]. To obtain *p* values for dose-response trends, we fitted ordinal exposure variables as continuous terms. Two sided *p* values are reported throughout this paper.

Results

Mean weight, height and BMI at baseline were higher for cases than for subcohort members (Table 2.1). Cases more often smoked than subcohort members: 44.7% of male cases and 24.2% of female cases smoked at baseline as compared with 36.8% of male subcohort members and 21.0% of female subcohort members (Table 2.1).
Results for height, weight, and non-occupational physical activity are shown stratified by sex. The interaction term for sex X height was statistically significant (p=0.02). Even though the interaction terms for weight and non-occupational physical activity were not statistically significant (p=0.20 and p=0.21, respectively), we decided to show results stratified by sex, since category limits for weight differed for men and women and estimates for nonoccupational physical activity pointed in a different directions for men and women.
Height was not associated with RCC risk in men (Table 2.2). In women, however, an increased risk of 1.23 per 5-cm increment (95% CI: 1.03-1.46) was observed (Table 2.2). Weight increased RCC risk approximately 10% per 5 kg for men and women (Table 2.2), while further adjustment for smoking did not materially change the risk estimates.
A high BMI was associated with increased risk of RCC (per 1-kg/m^2, rate ratio (RR): 1.05; 95% CI: 1.01-1.10), though the trend was not linear (Table 2.3). Additional adjustment for smoking did not alter the estimates (data not shown). After further adjustment for energy intake and physical activity, the increased risk of BMI remained (RR: 1.07; 95% CI: 1.02-1.12) (Table 2.3).
BMI at age 20 years was not related to RCC (Table 2.3). However, gaining body mass in adult life was associated with an increase in risk of RCC. A BMI gain of 1 kg/m^2 was associated with increased RCC risk (RR: 1.06; 95% CI: 1.01-1.10) (Table 2.3).

Table 2.1 Distribution of data on anthropometrical variables, energy-intake, physical activity and potential confounders among patients with renal cell carcinoma and subcohort members at baseline, Netherlands Cohort Study on Diet and Cancer, 1986–1995[a].

		Cases		Subcohort	
	Category	Mean (SD[b])	%	Mean (SD[b])	%
Exposure variables					
Height (cm) at baseline					
Men		176.6 (7.0)		176.4 (6.7)	
Women		166.9 (5.8)		165.1 (6.2)	
Weight (kg) at baseline					
Men		79.3 (9.8)		77.9 (9.5)	
Women		71.7 (10.8)		68.5 (10.3)	
Body mass index[c] at baseline		25.5 (3.1)		25.1 (3.1)	
Body mass index at age 20 years		21.7 (2.6)		21.5 (2.6)	
Change in body mass index (baseline minus age 20)		3.9 (3.3)		3.5 (3.4)	
Energy intake (kcal/day)					
Men		2140 (500)		2166 (511)	
Women		1652 (375)		1686 (397)	
Occupational physical activity	<8		59.1%		57.6%
(men only) (kJ/minute)	8-12		26.5%		26.5%
	>12		14.4%		16.0%
Nonoccupational physical activity (minutes/day)					
Men					
<30			22.8%		18.4%
30-60			24.0%		30.9%
60-90			17.0%		18.8%
>90			36.3%		31.9%
Women					
<30			26.4%		24.9%
30-60			31.0%		31.1%
60-90			24.1%		22.5%
>90			18.4%		21.4%
Confounding factors					
Age (years)		62.1 (3.9)		61.4 (4.2)	
Current cigarette smoker					
Men			44.7%		36.8%
Women			24.2%		21.0%
No. of cigarettes smoked per day					
Men		17.6 (12.7)		14.7 (11.4)	
Women		4.5 (7.6)		4.6 (7.7)	
No. of years of smoking					
Men		33.8 (14.0)		29.4 (15.9)	
Women		11.8 (16.0)		11.3 (15.9)	

[a] Analyses were based on 275 incident cases (179 men and 96 women) and 4,779 subcohort members (2,335 men and 2,444 women), though totals vary because of missing values. Data pertain to both sexes unless otherwise specified; [b] SD, standard deviation; [c] Weight (kg)/height(m)^2

Table 2.2 Rate ratios for renal cell carcinoma according to height and weight, Netherlands Cohort Study on Diet and Cancer, 1986–1995[a].

Variable	Categorical mean	No. of cases	No. of person-years in subcohort	RR[b,c]	95% CI[b]	RR[d]	95% CI
Height (cm) at baseline							
Men							
<170[e]	166.0	25	2,630	1		1	
170 - <175	171.9	38	4,802	0.81	0.47-1.37	0.72	0.42-1.25
175 - <180	176.8	57	5,689	1.00	0.59-1.70	0.87	0.50-1.54
180 - <185	181.7	31	3,892	0.72	0.40-1.29	0.70	0.38-1.28
>=185	188.0	22	2,310	0.75	0.39-1.43	0.71	0.36-1.39
p trend				0.37		0.38	
Continuous variable (per 5-cm increment)		173	19,322	0.96	0.84-1.11	0.97	0.84-1.13
Women							
<160[e]	155.7	10	3,582	1		1	
160 - <165	161.9	20	5,326	1.26	0.59-2.70	1.20	0.53-2.74
165 - <170	166.8	31	6,962	1.41	0.69-2.90	1.55	0.73-3.27
170 - <175	171.5	22	3,595	1.79	0.84-3.79	1.95	0.89-4.30
>=175	176.9	8	1,521	1.56	0.68-3.55	1.64	0.67-3.99
p trend				0.13		0.07	
Continuous variable (per 5-cm increment)		91	20,986	1.17	0.99-1.39	1.23	1.03-1.46
Weight (kg) at baseline							
Men							
<75[e]	68.5	55	7,055	1		1	
75 - <80	76.4	35	3,820	1.17	0.74-1.84	1.17	0.72-1.89
80 - <85	81.4	32	4,000	1.10	0.68-1.77	1.06	0.64-1.76
85 - < 90	86.2	21	2,130	1.35	0.78-2.32	1.29	0.73-2.29
>= 90	94.9	30	2,317	1.76	1.04-2.96	1.73	0.99-3.00
p trend				0.05		0.07	
Continuous variable (per 5-kg increment)		173	19,322	1.10	1.00-1.21	1.09	0.98-1.20
Women							
<65[e]	58.5	22	7,498	1		1	
65 - <70	66.6	19	4,351	1.29	0.69-2.40	1.18	0.62-2.26
70 - <75	71.4	14	3,748	1.08	0.55-2.12	1.10	0.56-2.17
75 - < 80	76.3	16	2,328	2.04	1.07-3.91	2.09	1.08-4.02
>= 80	86.1	20	3,060	1.91	1.06-3.47	1.54	0.82-2.92
p trend				0.01		0.05	
Continuous variable (per 5-kg increment)		91	20,986	1.13	1.02-1.24	1.11	1.01-1.23

[a] Height and weight were always entered into the model simultaneously. Numbers of cases and person-years in the subcohort were lower in the multivariable analyses because of missing values; [b] RR, rate ratio; CI, confidence interval; [c] Adjusted for age (continuous variable); [d] Adjusted for age (continuous variable) and smoking (current smoker: yes/no; number of cigarettes smoked per day: continuous variable; number of years of smoking: continuous variable); [e] Reference group

Table 2.3 Rate ratios for renal cell carcinoma according to BMI, [a] BMI at age 20 years, and change in BMI[a] between age 20 years and baseline, Netherlands Cohort Study on Diet and Cancer, 1986–1995[b].

Variable	Categorical mean	No. of cases	No. of person-years in subcohort	RR[a,c]	95% CI[a]	RR	95% CI
BMI[d] at baseline[e]							
<23	21.4	49	9,596	0.81	0.56-1.17	0.77	0.50-1.19
23 - < 25[f]	24.0	83	12,032	1		1	
25 - < 27	25.9	54	9,460	0.81	0.57-1.16	0.92	0.61-1.38
27 - < 30	28.2	62	6,503	1.40	0.99-1.97	1.46	0.97-2.21
>=30	32.3	16	2,717	1.03	0.59-1.80	1.04	0.54-1.99
p trend				0.05		0.04	
Continuous variable (per kg/m^2)		264	40,308	1.05	1.01-1.10	1.07	1.02-1.12
BMI at age 20 years[g]							
<20[f]	18.4	58	8,876	1		1	
20 - <21	20.5	28	5,559	0.67	0.42-1.08	0.76	0.47-1.23
21 - <23	22.0	70	10,964	0.88	0.61-1.26	0.96	0.66-1.41
>=23	24.7	72	9,062	1.11	0.78-1.59	1.18	0.80-1.72
p trend				0.41		0.29	
Continuous variable (per kg/m^2)		228	34,461	1.02	0.97-1.08	1.03	0.98-1.08
Change in BMI since age 20 years[g, h]							
<0	-2.0	14	3,735	0.51	0.29-0.90	0.50	0.27-0.90
0 - <4[f]	2.2	115	16,445	1		1	
4 - <8	5.7	74	11,170	1.04	0.76-1.43	1.02	0.74-1.42
>=8	10.2	24	3,002	1.53	0.96-2.46	1.31	0.79-2.19
p trend				0.01		0.04	
Continuous variable (per kg/m^2)		227	34,352	1.07	1.02-1.11	1.06	1.01-1.10

[a] BMI, body mass index; RR, rate ratio; CI, confidence interval; [b] Numbers of cases and person-years in the subcohort were lower in the multivariable analyses because of missing values. All data pertain to both sexes; [c] Adjusted for age (continuous variable) and sex (male/female); [d] Weight (kg)/height(m)2; [e] Multivariable rate ratios were adjusted for age (continuous variable), sex (male/female), smoking (current smoker: yes/no; number of cigarettes smoked per day: continuous variable; number of years of smoking: continuous variable), energy intake (continuous variable), non-occupational physical activity (<30, 30-60, 60-90, or >90 min/day), and, for men only, occupational physical activity (<8, 8-12, or >12 kj/min); [f] Reference group; [g] Multivariable rate ratios were adjusted for age (continuous variable), sex (male/female), and smoking (current smoker: yes/no; number of cigarettes smoked per day: continuous variable; number of years of smoking: continuous variable); [h] BMI at age 20 years was included in all models for BMI change.

Energy intake was not related to risk of RCC. Estimates did not change after adjustment for smoking or after further adjustment for BMI and physical activity (data not shown). In addition, we tested the highest quintile against the first quintile for energy intake. We did not observe an increased risk for persons in the highest quintile, either in the model with age, sex and physical activity (RR: 0.80; 95% CI: 0.50-1.27) or in the model additionally adjusted for smoking and BMI (RR: 0.83; 95% CI: 0.51-1.36).

For men, estimates for occupational physical activity greater than or equal to 8 kj/minute or nonoccupational physical activity greater than or equal to 30 minutes/day were all less than 1 (Table 2.4). Moreover, the risk for men was significantly decreased for nonoccupational physical activity of 30-60 minutes/day (RR: 0.52; 95% CI: 0.30-0.91), but there was no significant trend (p=0.63). Estimates for nonoccupational physical activity for women (Table 2.4) were mostly larger than one but never statistically significant.

Table 2.4 Rate ratios for renal cell carcinoma according to physical activity, Netherlands Cohort Study on Diet and Cancer, 1986–1995[a].

Variable	No. of cases	No. of person-years in subcohort	RR[b,c]	95% CI[b]	RR[d]	95% CI
Occupational physical activity (men only) (kJ/minute)						
< 8[e]	76	8,617	1		1	
8-12	35	3,905	0.98	0.64-1.49	0.86	0.54-1.39
>12	19	2,312	0.86	0.50-1.46	0.82	0.46-1.47
p trend			0.60		0.44	
Nonoccupational physical activity (minutes/day)						
Men						
<30[e]	34	2,580	1		1	
30-60	30	4,861	0.46	0.28-0.77	0.52	0.30-0.91
60-90	24	2,732	0.67	0.38-1.17	0.63	0.34-1.16
>90	42	4,661	0.66	0.41-1.07	0.74	0.44-1.23
p trend			0.47		0.63	
Women						
<30[e]	23	4,937	1		1	
30-60	27	6,234	0.95	0.54-1.68	1.13	0.59-2.15
60-90	21	4,444	1.07	0.59-1.94	1.43	0.73-2.79
>90	16	4,279	0.85	0.44-1.63	1.13	0.56-2.29
p trend			0.74		0.55	

[a] Numbers of cases and person-years in the subcohort were lower in multivariable analyses because of missing values. Occupational and nonoccupational physical activity were simultaneously entered into the model for men; [b] RR, rate ratio; CI, confidence interval; [c] Adjusted for age (continuous variable); [d] Multivariable rate ratios were adjusted for age (continuous variable), smoking (current smoker: yes/no; number of cigarettes smoked per day: continuous variable; number of years of smoking: continuous variable), energy intake (continuous variable), and body mass index (weight (kg)/height (m)^2); [e] Reference group.

Discussion

Our results suggest that a high BMI is an independent risk factor for RCC and that this relation is not influenced by energy intake and/or physical activity. Furthermore, energy intake does not seem to be an independent risk factor for RCC, while the role of physical activity remains unclear. BMI at age 20 years was not related to RCC risk, while BMI gain between young adulthood and baseline was associated with increased RCC risk. For women, height was associated with an increased risk of RCC.

Since RCC is relatively rare, most studies that have evaluated the relation between anthropometric measures and RCC risk have been case-control studies. Only four cohort studies have reported on the relation between anthropometric factors and RCC risk[10,13,15,41]. The prospective cohort setting makes selection bias in our study unlikely, while case-control studies, especially hospital-based case-control studies, may suffer from selection and information bias. In the current study, the estimated rate ratio for the association between BMI and RCC was 1.07, based on 264 incident cases. This is in line with the findings of a meta-analysis on 14 studies[1] and the results of other cohort studies reporting on BMI and RCC risk[10,13,15,41]. Only one hospital-based case-control study reported no association between BMI and RCC risk[42]. Height and weight were self-reported in our study, as in most other studies, which means misclassification might be present. Systematic underestimation of weight and overestimation of height have been reported[43,44]. More specifically, the higher the measured BMI, the greater the underestimation of weight and the overestimation of height for men and women[44]. This tendency could have lead to underestimation of the effect of BMI on RCC risk in our study.

An increased risk of RCC with increasing BMI has been reported consistently. This observation may fit both suggested biological mechanisms, since it has been reported that obese persons have higher levels of free IGF-I[22] and exhibit increased lipid peroxidation[29]. Both have been linked to tumor development. In view of the strong relation between BMI and RCC, it is remarkable that no convincing mechanism has yet been proposed for this relation. Further research is needed.

In this study, we found RCC risk to increase with height (contradictory to most other studies on height and RCC) in women. An increased cancer risk with height has been observed before for women in the Netherlands Cohort Study on Diet and Cancer for breast cancer[45] and ovarian cancer[46]. However, for men, an increased risk with height was not observed in the current study or in a study of prostate cancer carried out within the Netherlands Cohort Study on Diet and Cancer[47]. Biological mechanisms through which height and cancer risk are linked are not clear yet, but IGF-I is known to play a fundamental role in somatic growth[48]. A real albeit relatively weak association between height and risk of several cancers possibly exists, but the relation has been unclear for RCC because research on this topic is scarce[48]. It is unclear to us why sex would modify the association between height and RCC. As far as we know, no other studies report differences between men and women.

Another cohort study found weight at age 18 years and weight gain since age 18 to be independent risk factors for women[15]. Our results on BMI at age 20 and BMI change between young adulthood and baseline do not support the hypothesis that BMI at young adulthood is an independent risk factor of RCC, but BMI gain might be. However, BMI at age 20 and BMI gain between age 20 and baseline were retrospectively reported, which means recall bias might have been present. However, the effect of the recall bias will be nondifferential since only incident cases are included in the analyses, and would thus result in attenuated RRs for these factors.

Only the International Renal Cell Cancer study[19], a large case-control study, reported a positive association between energy intake and RCC risk, but this association might have been due to recall bias. In the current study, no association was observed between energy intake and RCC. This is in line with the findings of another cohort study[15]. This is remarkable, since a high BMI is associated with an increased risk of RCC and a high BMI results from excess energy intake in relation to energy expenditure[22]. This might be explained by the fact that energy intake tends to be underreported by overweight persons[49]. It might also be possible that measurement error concealed a possible effect of energy intake on RCC. The validity of reported energy intake in our study was checked by comparing results of the food frequency questionnaire with 3-day diaries completed at three time points during a calendar year. Reported intakes were, on average, 300 kcal lower than those reported by means of the diaries. However, the questionnaire was able to rank subjects according to their energy intake[34]. Energy intake does not seem to be an independent risk factor for RCC in the current study or in the other cohort study that reported on energy intake, but replication, preferably by other cohort studies, is desirable.

We did not find a clear association of physical activity and risk of RCC. Results of other studies are also inconsistent[16,20-22,50]. In general, there has been little standardization of the methods used for assessing physical activity in epidemiological studies, and few methods have been appropriately tested for reliability and validity. The use of crude measures of physical activity is likely to result in measurement error and difficulty in determining the true nature of the relation between physical activity and cancer risk[51]. Our measures for nonoccupational physical activity might have been affected by nondifferential misclassification, since it is socially desirable to be active. Thus, persons engaging in little or no activity may have overestimated their nonoccupational physical activity. The result of nondifferential misclassification is hard to predict. Our findings might be an underestimation of the real effect, but it is also possible that the effect in one of the categories was overestimated. An inverse association of leisure-time physical activity with obesity and mean BMI in men and women has been reported[52], which would suggest that the risk of RCC should be decreased for higher levels of (leisure-time) physical activity. Our results point in the direction of a possible protective effect of physical activity on risk of RCC for men, though not as clearly as the results reported by Mahabir *et al.*[21]. The study by Mahabir *et al.* was restricted to male smokers; thus, its results not be generalizable. Furthermore,

there were only five cases in the highest leisure-time physical activity category, and men with a higher level of recreational physical activity showed a healthier lifestyle (i.e., smoked less)[21]. A more standardized manner for investigating the role of physical activity in large study groups might contribute to an unraveling of the role of physical activity.

In summary, our results confirm an increased risk of RCC with BMI, while BMI gain between young adulthood and baseline may also increase RCC risk. An effort should be undertaken to elucidate possible underlying mechanisms between factors such as BMI, BMI gain in adulthood, physical activity and energy intake and cancer risk, specifically RCC risk.

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3

Vegetable and fruit consumption and risk of renal cell carcinoma: results from the Netherlands cohort study

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Abstract

Vegetable and fruit consumption is generally inversely associated with various cancer types, including Renal Cell Carcinoma (RCC). The Netherlands cohort study on diet and cancer (NLCS) consists of 120,852 men and women, aged 55-69, who filled-out a self-administered questionnaire that includes a 150-item food-frequency questionnaire and additional questions on lifestyle factors at baseline in 1986. A case-cohort approach was used. After 9.3 years of follow-up, 275 microscopically confirmed incident cases were identified. Subjects with incomplete or inconsistent dietary data were excluded, leaving 260 RCC cases for analyses on fruit consumption and 249 RCC cases for analyses on vegetable consumption. Incidence rate ratios (RR) and corresponding 95% confidence intervals (CI) were estimated using Cox proportional hazard models. RRs for exposure variables are expressed per increment of 25 g/day and are adjusted for age, sex, smoking, body mass index and history of hypertension at baseline. The RRs for vegetable consumption were further adjusted for fruit consumption and vice versa. Total vegetable and fruit consumption (RR: 1.00; 95% CI: 0.97-1.02), vegetable (RR: 1.00; 95% CI: 0.96-1.06) and fruit consumption (RR: 1.00; 95% CI: 0.97-1.03) were not associated with RCC risk. Also, no association existed for botanical subgroups of vegetables and fruit. For 30 individual vegetables and fruits, we observed one that significantly increased risk (mandarin consumption, RR: 1.76; 95% CI: 1.28-2.42), which must be regarded cautiously because of multiple testing. These results suggest the absence of an association between vegetable and/or fruit consumption and RCC risk.

Introduction

In general, vegetable and fruit consumption is assumed to reduce risk of various cancer types. Most reviews on Renal Cell Carcinoma (RCC) also conclude that vegetable and fruit consumption may reduce RCC risk[1-5]. Statistically significant reduced risks for RCC have been reported for the highest tertile of vegetable and fruit consumption[6], for vegetable and fruit consumption but restricted to men[7], for cruciferous/dark green vegetables[8], for vegetable and vegetable juice consumption[9], for carrot consumption[10] for root vegetable and banana consumption[11] and for fruit consumption[12,13]. Furthermore, two studies showed estimates pointing in the direction of a protective effect, but associations were not statistically significantly different from one[14,15]. Finally, three studies observed null associations[16-18].
Antioxidant vitamins, fibres or enzyme inducers present in vegetable and fruits might be responsible for this preventive effect[5]. Plants rich in nitrosation inhibitors, antioxidants or enzyme inducers, e.g., ascorbate and polyphenols or carotenoid-rich vegetables, garlic and cruciferous vegetables may thus be most effective in preventing cancer[5]. The biological plausibility that vegetable and fruit consumption reduces cancer risk is present, but so far reported results do not support this notion unequivocally[6-18].
We investigated total vegetable and fruit consumption, as well as the consumption of botanical groups of vegetables and fruits and individual vegetables and fruits and RCC risk in a large cohort. For some important risk factors stratified analyses will be carried out, e.g. for smoking because smoking increases oxidative stress and a more pronounced protective effect has been reported for never smokers[12,15].

Materials and methods

Netherlands Cohort Study

The Netherlands Cohort Study on diet and cancer is a large cohort study that started in September 1986. The study design has been reported in detail elsewhere[19]. Briefly, the cohort included 120,852 men and women aged 55-69 years in 1986. An elderly cohort was selected because dietary habits (and their contrasts) are stabilized, and such a cohort will yield sufficient cases for meaningful analyses within a reasonable time period[19]. The case-cohort design was used, which means that a subcohort of 5,000 men and women was randomly sampled from the cohort after baseline exposure measurement to estimate the number of person-years for the entire cohort, whereas cases were enumerated for the entire cohort[20].

Follow-up for incident cancers and vital status

The entire cohort was followed for incident cancer by computerized record linkage with the Netherlands Cancer Registry and PALGA, a national database of pathology

reports. The method of record linkage to obtain information on cancer incidence has been described previously[21]. The completeness of cancer follow-up was estimated to be more than 96%[22]. After 9.3 years of follow-up 275 incident RCC cases have been identified.
The subcohort has been followed up for vital status information biennially by mail. The vital status of subcohort members who did not respond was completed by contacting municipal population registries. Only two male subcohort members were lost to follow-up after 9.3 years of follow-up and were censored. Subcohort members with prevalent cancer at baseline (other than skin cancer) were excluded from analyses, leaving 4,779 subcohort members.

Questionnaire

At baseline, all cohort members completed a mailed, self-administered questionnaire on dietary habits, lifestyle, smoking, personal and family history of cancer and demographic data. The dietary section of the questionnaire was a 150-item semi-quantitative food-frequency questionnaire, which was validated against 3-day diaries completed at three time points during a calendar year[23]. The questionnaire concentrated on the habitual consumption of food and beverages during the year preceding the start of the study. With regard to vegetable consumption, participants were asked to report their frequency of consumption of a number of vegetables, both in summer and in winter. They could choose one of six categories, ranging from "never or less than once a month" to "three to seven times per week". Usual serving sizes were asked for string beans and cooked endive only, the mean of which served as an indicator for serving sizes of all cooked vegetables. This procedure was chosen because in a pilot study (based on an extensive dietary history with food models and photos used to estimate individual portion sizes) it was shown that serving sizes of different types of cooked vegetables were correlated within subjects[24]. To derive an individual serving size for each type or vegetable the indicator serving size was multiplied with a type-specific factor calculated from the same pilot study data as the ratio of the means of the specific to the indicator serving sizes[24]. For tomatoes and sweet peppers, consumption was asked in pieces per week and month, respectively, during summer and winter. With regard to fruit consumption, frequencies varying from "never or less than once a month" to "six or seven days per week" and amounts consumed could be reported for mandarins, oranges, grapefruits, grapes, bananas, apples/pears and strawberries. Using standard portion sizes, these frequencies and amounts have been converted to consumption in grams per day. The choice of items for inclusion in the questionnaire was such that it covered almost all vegetables and fruits eaten regularly, with the exceptions of chicory, red cabbage and cucumber. Broccoli was a rarely available vegetable in 1986 and therefore not included. However, an open-ended question on other foods eaten on a regular basis was included. Participants could write down how many times per week they ate such a food and how much they were used to eat on each occasion.

According to criteria published before[23], subjects with incomplete or inconsistent dietary data were excluded; 260 RCC cases and 4,441 subcohort members remained for analyses on fruit consumption. In addition, we computed an error index based on the consistency of responses on vegetable questions. Questions on vegetable consumption appeared early in the questionnaire, which led some subjects to making mistakes on these particular items, while items appearing further along in the questionnaire were filled out without problems. When the vegetable error index exceeded a certain value, *i.e.,* more than three errors, subjects were excluded from analyses on vegetable consumption. Therefore, data analysis regarding vegetable consumption was based on 249 RCC cases and 4,201 subcohort members.

Data analysis

Rate ratios (RRs) were calculated for total vegetable and fruit consumption, total vegetable consumption, total fruit consumption, cooked vegetable consumption and raw vegetable consumption. RRs were also calculated for botanical groups of vegetables and fruits (composition of botanical groups is shown in Appendix 1), with the exception of groups based on one main constituent (carrots, beets, tomatoes, grapes, bananas and strawberries). These were analysed in the individual vegetable and fruit analysis only. RRs were calculated per increment of 25 g/day. Also, subjects were classified into quintiles and tertiles of vegetable or fruit consumption, based on the distribution in the subcohort. Analyses for total vegetable and fruit consumption, total vegetable and total fruit consumption have been repeated excluding the first two years of follow-up to evaluate whether pre-clinical RCC influenced results.

Based on the literature and previous analyses, considered confounders were age (continuous), sex, cigarette smoking (current smoker yes or no, number of smoking years and number of cigarettes a day), alcohol intake, body mass index (BMI), history of hypertension, physical activity, energy intake and social economic status (SES) based on education. We did not adjust for family history of RCC (present or not in a first-degree relative) since only 49 participants (4 cases and 45 subcohort members) reported a first-degree relative with RCC. Age and sex were included in all analyses. Factors that statistically significantly contributed to the model were entered in the multivariable model, leaving BMI and a history of hypertension. Smoking was also entered since some of the smoking variables to describe the smoking status satisfied this criterion as well. Moreover, these factors are well known risk factors of RCC, and may be associated with a "healthy" lifestyle, which may also be associated with vegetable and fruit consumption. For all analyses on vegetable consumption, fruit consumption was also included as a confounder and vice versa. We investigated possible interaction by sex by entering an interaction term in the model and assessing the significance of this term using the Wald test. Since no interaction on RCC risk between sex and dietary intakes was observed, results are shown for men and women combined. Furthermore, total vegetable and fruit consumption, vegetable consumption

and fruit consumption are presented stratified by smoking status (never, ex- or current smoker), BMI (<25 kg/m^2 or ≥25 kg/m^2) and a history of hypertension (yes or no).
RRs and corresponding 95 percent confidence intervals (CI) for RCC were estimated using Cox proportional hazard models processed with the STATA statistical software package (STATA statistical software, Release 7, STATA Corporation, College Station, TX, USA, 2001) after testing the proportional hazards assumption using scaled Schoenfeld residuals[25]. The proportional hazards assumption was not rejected. Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort[26]. To obtain *p* values for dose-response trends, ordinal exposure variables were fitted as continuous terms. Two sided *p* values are reported throughout this paper.

Results

The mean age (standard deviation) was 62.1 (3.9) for cases and 61.4 (4.2) for subcohort members. Sixty-five % of cases were male, compared to 49% of subcohort members. The mean BMI was somewhat higher for cases than for subcohort members (25.5 kg/m^2 compared to 25.1 kg/m^2). Almost a third (30%) of the cases and approximately a quarter (26%) of subcohort members reported a history of hypertension.
Also, cases were more often current smokers (38% compared to 29%) or ex-smokers (38% compared to 35%) and smoked more and longer than subcohort members (within the strata of ex- and current smokers).
No statistically significant interaction was shown for sex and total vegetable and fruit consumption (*p* value=0.40), for sex and vegetable consumption (*p* value=0.12) or for sex and fruit consumption (*p* value=0.99), so RRs for RCC were calculated for men and women combined.
Differences in vegetable consumption between cases and subcohort members were small (Table 3.1). However, mean fruit consumption was somewhat lower for cases than for subcohort members and this difference was present in all fruit groups (Table 3.1).
Table 3.2 shows means of vegetable and fruit consumption for never, ex- and current smokers, for a BMI<25 and ≥25 and for a positive history of hypertension or not (Table 3.2). Similar means were observed for vegetable consumption in different smoking groups and for strata of BMI and a history of hypertension. Mean fruit consumption was higher in never smokers. In the stratum of current smokers, cases ate more vegetables and less fruit than subcohort members. Among never smokers, cases consumed less fruit than subcohort members (Table 3.2). Fruit consumption was somewhat lower for cases with a BMI<25 (Table 3.2).

Table 3.1 Mean daily vegetable and fruit consumption among RCC cases and subcohort members at baseline, Netherlands Cohort Study on Diet and Cancer, 1986–1995.

Exposure variables	Cases Mean (SD)	Subcohort Mean (SD)
Total vegetables & fruit[a]	350.5 (141.3)	362.1 (151.9)
Total vegetables[a]	190.4 (76.7)	188.4 (75.6)
Cooked vegetables[a]	152.4 (65.3)	149.3 (61.3)
Raw vegetables[a]	38.0 (23.7)	39.1 (29.6)
Legumes[a]	36.1 (25.0)	32.4 (22.1)
Brassicas[a]	32.9 (20.2)	31.7 (19.8)
Leafy vegetables, cooked[a]	21.9 (15.5)	21.3 (15.8)
Leafy vegetables, raw[a]	9.1 (7.9)	10.0 (9.0)
Allium vegetables, cooked[a]	29.6 (24.8)	29.1 (24.3)
Total fruit[b]	161.9 (112.2)	175.3 (119.4)
Citrus fruit[b]	73.0 (71.4)	77.0 (74.3)
Apples, pears[b]	81.4 (74.6)	87.3 (82.4)

[a] Based on 249 incident cases and 4,201 subcohort members; [b] Based on 260 incident cases and 4,441 subcohort members

Multivariable rate ratios of RCC for total vegetable and fruit consumption as well as for botanical subgroups of vegetable and fruit consumption are shown in Table 3.3. We also analysed age and sex adjusted rates, but these were not very different (data not shown). "All vegetables and fruits", "all vegetables" and "all fruits" were not (inversely) associated with RCC risk (all RRs equalled 1). RRs of RCC for these groups hardly changed after exclusion of the first two years of follow-up (data not shown).

Legume consumption was associated with an increased risk for every 25 grams of legume consumed more per day (multivariable adjusted RR: 1.14; 95% CI: 0.99-1.33) (Table 3.3). This increased risk was restricted to the highest quintile of legume consumption (RR: 1.31; 95% CI: 0.83-2.07). An increment of 25 gram per day of raw, leafy vegetables more was associated with a reduced RCC risk (RR: 0.68; 95% CI: 0.45-1.04), with an indication of a decreasing risk with increasing consumption (p value for trend: 0.11) (Table 3.3).

Table 3.4 shows RRs of RCC for individual vegetables and fruits. No statistically significant reduced or increased risks were observed for individual vegetables. A significantly increased risk of RCC was observed for mandarin consumption (RR: 1.76; 95% CI: 1.28-2.42). Other citrus fruits, however, were not associated with either an increased or decreased risk of RCC (Table 3.4). Banana consumption may be associated with a reduced RCC risk (RR: 0.85; 95% CI: 0.72-1.01).

Table 3.2 Mean daily vegetable and fruit consumption among RCC cases and subcohort members at baseline, according to cigarette smoking (never, ex- or current smoker), BMI (<25 and ≥25) and history of hypertension reported (yes or no), Netherlands Cohort Study on Diet and Cancer, 1986-1995.

	Vegetable consumption			
	Cases (N)	Cases Mean (sd)	Subcohort members (N)	Subcohort Mean (sd)
Never smoker	61	183.9 (71.4)	1,496	187.4 (74.6)
Ex-smoker	92	186.5 (73.4)	1,525	192.9 (74.5)
Current smoker	96	198.4 (82.8)	1,180	183.8 (78.2)
BMI<25	119	187.3 (72.7)	2,221	188.3 (76.4)
BMI>=25	130	193.4 (80.4)	1,980	188.4 (74.8)
No history of hypertension	172	187.7 (79.5)	3,118	186.9 (74.7)
History of hypertension	77	196.6 (70.0)	1,083	192.6 (78.3)
	Fruit consumption			
	Cases (N)	Cases Mean (sd)	Subcohort members (N)	Subcohort Mean (sd)
Never smoker	63	188.2 (113.6)	1,588	202.5 (123.3)
Ex-smoker	100	172.2 (106.3)	1,594	173.4 (117.1)
Current smoker	97	134.3 (112.5)	1,259	143.3 (108.7)
BMI<25	124	151.4 (99.3)	2,344	175.9 (119.5)
BMI>=25	136	171.5 (122.4)	2,097	174.5 (119.4)
No history of hypertension	180	161.0 (115.0)	3,290	173.2 (119.7)
History of hypertension	80	164.0 (106.5)	1,151	181.3 (118.4)

Table 3.5 shows multivariable adjusted RRs for vegetable and fruit consumption, stratified by smoking status, BMI and history of hypertension. The estimated RRs did not differ largely between strata. None of the interaction terms of smoking, BMI or history of hypertension with tertiles of total vegetable and fruit consumption, with total vegetable consumption or with total fruit consumption were statistically significant.

Table 3.3 Multivariable adjusted rate ratios and 95% confidence intervals for RCC for total and subgroups of vegetable and fruit consumption, Netherlands Cohort Study on Diet and Cancer, 1986-1995.

Vegetable/Fruit group	Quintile of consumption					*p* value for trend	Continuous / 25g per day
	1 (low)[a]	2	3	4	5 (high)		
All vegetables and fruits							
Median consumption (g/day)	189	275	343	418	556		
Cases of RCC	50	34	48	51	39		222
Person-years	6,601	6,727	7,016	6,789	6,979		34,111
Multivariable adjusted RR[b]	1	0.69	0.94	1.01	0.78	0.79	1.00
95% CI	-	0.44-1.09	0.62-1.42	0.67-1.52	0.50-1.21		0.97-1.02
All vegetables							
Median consumption (g/day)	104	145	178	217	287		
Cases of RCC	48	44	38	50	42		222
Person-years	6,587	6,933	6,888	6,831	6,873		34,111
Multivariable adjusted RR[2]	1	0.87	0.77	1.04	0.84	0.76	1.00
95% CI	-	0.57-1.34	0.49-1.19	0.68-1.59	0.54-1.31		0.96-1.06
Cooked vegetables[c]							
Median consumption (g/day)	79	114	142	173	230		
Cases of RCC	48	40	42	42	50		222
Person-years	6,622	6,802	7,012	6,848	6,827		34,111
Multivariable adjusted RR[b]	1	0.79	0.78	0.81	0.97	0.24	1.01
95% CI	-	0.51-1.23	0.51-1.21	0.53-1.25	0.63-1.48		0.95-1.07
Raw vegetables[3]							
Median consumption (g/day)	8	22	34	48	76		
Cases of RCC	33	47	50	52	40		222
Person-years	6,753	6,802	6,770	6,905	6,881		34,111
Multivariable adjusted RR[b]	1	1.46	1.64	1.63	1.26	0.88	0.99
95% CI	-	0.91-2.33	1.04-2.59	1.04-2.58	0.78-2.06		0.89-1.09
Legumes[d]							
Median consumption (g/day)	10	19	28	38	60		
Cases of RCC	44	39	41	40	58		222
Person-years	6,586	6,976	6,761	6,898	6,890		34,111
Multivariable adjusted RR[b]	1	0.86	0.92	0.91	1.31	0.27	1.14
95% CI	-	0.54-1.38	0.58-1.46	0.56-1.49	0.83-2.07		0.99-1.33
Brassicas[d]							
Median consumption (g/day)	10	20	28	38	57		
Cases of RCC	43	34	51	52	42		222
Person-years	6,627	6,819	6,915	6,847	6,904		34,111
Multivariable adjusted RR[b]	1	0.78	1.12	1.18	0.90	0.81	0.98
95% CI	-	0.49-1.26	0.72-1.73	0.74-1.88	0.54-1.50		0.80-1.20

▶

Vegetable/Fruit group	Quintile of consumption					*p* value for trend	Continuous / 25g per day
	1 (low)[a]	2	3	4	5 (high)		
Leafy vegetables, cooked[d]							
Median consumption (g/day)	4	12	19	27	41		
Cases of RCC	41	48	46	41	46		222
Person-years	6,763	6,861	6,835	6,806	6,847		34,111
Multivariable adjusted RR[b]	1	1.17	1.15	1.01	1.10	0.98	0.94
95% CI	-	0.74-1.83	0.72-1.82	0.61-1.65	0.67-1.80		0.75-1.19
Leafy vegetables, raw[d]							
Median consumption (g/day)	2	4	7	12	22		
Cases of RCC	54	45	42	43	38		222
Person-years	7,836	5,759	6,876	6,736	6,905		34,111
Multivariable adjusted RR[b]	1	1.04	0.83	0.84	0.71	0.11	0.68
95% CI	-	0.68-1.60	0.54-1.29	0.54-1.30	0.45-1.11		0.45-1.04
Allium vegetables, cooked[d]							
Median consumption (g/day)	5	16	24	37	60		
Cases of RCC	65	31	45	38	43		222
Person-years	9,447	4,187	6,939	6,797	6,742		34,111
Multivariable adjusted RR[b]	1	1.18	0.90	0.74	0.84	0.16	1.01
95% CI	-	0.75-1.85	0.60-1.36	0.48-1.14	0.55-1.30		0.87-1.17
All fruits							
Median consumption (g/day)	44	107	155	215	326		
Cases of RCC	47	45	51	39	50		232
Person-years	6,985	7,156	7,156	7,377	7,346		36,021
Multivariable adjusted RR[b]	1	0.98	1.15	0.84	1.08	0.58	1.00
95% CI	-	0.64-1.50	0.76-1.74	0.54-1.30	0.71-1.66		0.97-1.03
Citrus fruit[e]							
Median consumption (g/day)	3	26	60	98	176		
Cases of RCC	43	40	63	35	51		232
Person-years	7,092	7,275	7,206	7,195	7,253		36,021
Multivariable adjusted RR[b]	1	0.94	1.48	0.84	1.22	0.51	1.01
95% CI	-	0.60-1.47	0.98-2.26	0.52-1.36	0.79-1.89		0.97-1.06
Apples and pears[e]							
Median consumption (g/day)	6	40	80	116	181		
Cases of RCC	45	42	59	42	44		232
Person-years	7,151	7,154	7,375	6,995	7,346		36,021
Multivariable adjusted RR[b]	1	0.97	1.37	1.06	1.04	0.71	1.00
95% CI	-	0.63-1.51	0.91-2.06	0.67-1.67	0.67-1.62		0.96-1.04

[a] reference category; [b] Multivariable models include adjustment for age, sex, current smoker (yes/no), number of cigarettes per day, number of smoking years, BMI, history of hypertension and fruit or vegetable consumption for vegetable or fruit consumption, respectively; [c] Cooked and raw vegetables are simultaneously entered in the model; [d] Legumes, brassicas, cooked leafy vegetables, raw leafy vegetables, allium vegetables, carrots, beets, tomatoes and other cooked and raw vegetables are simultaneously entered in the model; [e] Citrus fruit, apples and pears, grapes, bananas, strawberries and other fruits and fruit juices are simultaneously entered in the model.

Table 3.4 Rate ratios and 95% confidence intervals for RCC for individual vegetable and fruit consumption, Netherlands Cohort Study on Diet and Cancer, 1986–1995.

Variable[a]	Median consumption (grams/day)	Age and sex adjusted RR per 25 g increment	95% CI	Multivariable adjusted RR[b] per 25 g increment	95% CI
Brussels sprouts	7	1.16	0.77-1.77	1.03	0.65-1.65
Leek	6	0.94	0.64-1.37	0.92	0.61-1.40
Sauerkraut	5	1.46	0.75-2.84	1.72	0.86-3.44
Cauliflower	12	0.93	0.64-1.36	0.89	0.60-1.32
Cabbage	5	0.81	0.50-1.33	0.87	0.51-1.47
Spinach	8	0.80	0.53-1.20	0.79	0.51-1.22
Endive prepared	10	1.21	0.90-1.63	1.07	0.78-1.47
Red beets	6	0.84	0.53-1.32	0.95	0.60-1.49
Carrots prepared	7	0.98	0.65-1.47	0.92	0.60-1.43
String and sliced beans	17	1.16	0.94-1.43	1.17	0.94-1.46
Broad beans	1	1.20	0.78-1.87	1.06	0.66-1.68
Kale	2	1.63	0.64-4.12	1.74	0.66-4.61
Endive raw	0	0.60	0.25-1.43	0.63	0.25-1.60
Lettuce	6	0.82	0.50-1.33	0.77	0.46-1.30
Carrots raw	0	0.89	0.60-1.31	0.93	0.61-1.41
Rhubarb	0	1.03	0.50-2.12	1.09	0.52-2.28
Apple sauce	4	0.98	0.79-1.22	0.98	0.78-1.24
Onions	11	1.11	0.92-1.33	1.08	0.88-1.32
Tomatoes	19	1.13	0.99-1.29	1.11	0.97-1.28
Mushrooms	4	0.82	0.32-2.08	0.82	0.29-2.34
Sweet peppers	1	0.58	0.22-1.54	0.72	0.28-1.87
Gherkins	0	0.76	0.49-1.18	0.71	0.42-1.21
Raisins	0	0.34	0.04-2.58	0.43	0.05-3.35
Mandarins	2	1.77	1.31-2.40	1.76	1.28-2.42
Oranges	32	1.00	0.95-1.06	1.02	0.96-1.08
Grapefruit	0	0.96	0.83-1.12	0.97	0.83-1.14
Grapes	1	0.77	0.53-1.12	0.73	0.49-1.09
Bananas	4	0.87	0.75-1.01	0.85	0.72-1.01
Apples, pears	53	0.99	0.95-1.03	1.00	0.96-1.04
Strawberries	5	0.88	0.57-1.37	0.99	0.65-1.50

[a] All vegetable variables (brussels sprouts through gherkins) are entered simultaneously in the model; rate ratios are based on 249 cases and 36,886 person-years. All fruit variables (raisins through strawberries) are entered simultaneously in the model; relative risks are based on 260 cases and 38,994 person-years. The number of cases and person years in the subcohort is lower in multivariable analyses due to missing values; [b] Multivariable models include adjustment for age, sex, current smoker (yes/no), number of cigarettes per day, number of smoking years, BMI, history of hypertension and fruit or vegetable consumption for vegetable or fruit consumption, respectively.

Table 3.5 Rate ratios and 95% confidence intervals for RCC for vegetable and fruit consumption, according to cigarette smoking (never, ex- or current smoker), BMI (<25 and ≥25) and history of hypertension reported (yes or no), Netherlands Cohort Study on Diet and Cancer, 1986–1995.

	No of cases / person-years in subcohort	Tertile of vegetable and fruit consumption			*p* value for trend	Continuous per 25 g increment
		1 (low)	2	3 (high)		
Vegetable consumption						
Never smoker[a]	59/12,970	1 (ref)	0.92 (0.47-1.77)	1.11 (0.57-2.18)	0.77	0.99 (0.91-1.09)
Ex-smoker[b]	82/12,221	1 (ref)	0.91 (0.52-1.59)	0.84 (0.48-1.49)	0.56	0.96 (0.87-1.05)
Current smoker[b]	81/8,921	1 (ref)	0.95 (0.53-1.71)	1.24 (0.72-2.15)	0.44	1.05 (0.98-1.13)
BMI<25[c]	112/18,673	1 (ref)	0.84 (0.52-1.36)	1.08 (0.67-1.73)	0.77	1.00 (0.94-1.07)
BMI≥25[c]	117/16,564	1 (ref)	1.00 (0.63-1.59)	1.04 (0.65-1.66)	0.87	1.01 (0.94-1.08)
History of hypertension = no[d]	151/25,241	1 (ref)	0.86 (0.57-1.31)	1.01 (0.67-1.51)	0.98	1.00 (0.94-1.06)
History of hypertension = yes[d]	71/8,870	1 (ref)	1.02 (0.54-1.92)	1.07 (0.56-2.04)	0.83	1.01 (0.93-1.08)
Fruit consumption						
Never smoker[a]	61/13,755	1 (ref)	0.76 (0.40-1.41)	0.71 (0.38-1.33)	0.30	0.98 (0.93-1.04)
Ex-smoker[b]	90/12,734	1 (ref)	0.94 (0.54-1.63)	1.21 (0.71-2.06)	0.46	1.01 (0.97-1.05)
Current smoker[b]	81/9,532	1 (ref)	1.17 (0.69-1.99)	0.79 (0.43-1.46)	0.54	0.99 (0.93-1.05)
BMI<25[c]	117/19,698	1 (ref)	0.97 (0.61-1.53)	0.81 (0.50-1.31)	0.39	0.97 (0.93-1.01)
BMI≥25[c]	122/17,527	1 (ref)	0.85 (0.53-1.34)	0.95 (0.60-1.49)	0.82	1.01 (0.97-1.06)
History of hypertension = no[d]	159/26,597	1 (ref)	1.04 (0.70-1.55)	0.96 (0.63-1.44)	0.83	1.01 (0.97-1.04)
History of hypertension = yes[d]	73/9,423	1 (ref)	0.81 (0.44-1.46)	0.88 (0.49-1.57)	0.67	0.98 (0.93-1.03)

[a] Rate ratios are adjusted for age, sex, BMI, history of hypertension and fruit or vegetable consumption for vegetable or fruit consumption, respectively; [b] Rate ratios are adjusted for age, sex, number of cigarettes smoked per day, years of cigarette smoking, BMI, history of hypertension and fruit or vegetable consumption for vegetable or fruit consumption; [c] Rate ratios are adjusted for age, sex, current smoker (yes/no), number of cigarettes smoked per day, years of cigarette smoking, history of hypertension and fruit or vegetable consumption for vegetable or fruit consumption, respectively; [d] Rate ratios are adjusted for age, sex, current smoker (yes/no), number of cigarettes smoked per day, years of cigarette smoking, BMI and fruit or vegetable consumption for vegetable or fruit consumption, respectively.

Discussion

Neither total vegetable and/or fruit consumption, nor consumption of a botanical group of vegetables or fruits, nor individual vegetables or fruits was associated with a decreased RCC risk in this cohort study. The only statistically significant result observed in 30 analyses of specific vegetables and fruits was an increased risk for mandarin consumption, which must be regarded cautiously because of multiple testing. Also, no modifying effect for smoking, BMI or a history of hypertension could be shown.

The prospective nature of a cohort study together with the completeness of follow-up, as has been achieved in this study, reduced the potential for selection bias to a minimum. Information bias, *i.e.,* a change in (report of) dietary habits of RCC cases due to the disease, is also largely avoided in a prospective study because dietary habits were reported before the disease was diagnosed. A change in dietary habits of subjects with pre-clinical RCC at the time of completing the baseline questionnaire remains possible. An indicator of advanced disease such as weight loss, is estimated to be associated with RCC in approximately 35% of cases[27]. Weight loss may be induced by substances such as cytokines, insulin, inflammatory mediators etc. produced in pathologic amounts by the tumour[27]. However, results excluding the first two years of follow-up did not differ from presented results (including the first two years of follow-up), indicating that our results were not influenced by the possible presence of pre-clinical RCC cases. Also, more than 50% of RCCs are now detected incidentally because of the more pervasive use of non-invasive imaging for the evaluation of a variety of non-specific symptom complexes[27].

Residual confounding by risk factors for RCC such as smoking or BMI is a realistic threat. Clustering of low vegetable and fruit consumption with smoking has been reported for the Dutch population[28], and insufficient control of one factor will then confound the association between the other factor and RCC. We tried to model cigarette smoking habits such that they best explained RCC risk, resulting in a model including the habitual number of cigarettes smoked per day (smoking amount) and the number of years smoked (smoking duration), both as continuous variables. Furthermore, estimated RRs were not different for smoking strata (never, ex, current).

Correction for BMI may have influenced results because high vegetable and fruit consumption might be associated with a lower BMI (as a result of the dietary pattern). However, the correlation of BMI with total vegetable and fruit consumption is not large ($r=0.025$; p value=0.0781). Also, mean BMI did not differ for quintiles of total vegetable and fruit consumption, vegetable consumption and fruit consumption (mean BMI range: 24.8–25.2 kg/m^2). Moreover, the RR and corresponding 95% CI for vegetable and fruit consumption did not change after removing BMI from the model.

Although we measured vegetable and fruit consumption extensively, a potential limitation remains misclassification of exposure. Usual vegetable consumption is not very easy to assess in food-frequency questionnaires (or in other methods of dietary

assessment), particularly if portion sizes have to be estimated. In the NLCS validation study, the FFQ was tested against 3-day diaries completed at three time points during a calendar year among 212 randomly selected subcohort members. The correlation coefficient for total vegetables consumption was 0.4[23], which is moderate, but comparable to the figure reported for many other prospective studies[29,30]. One of the reasons for the low correlation may be the relative lack of true contrast in the frequency of vegetable consumption in a population such as the Dutch, because people are accustomed to a diet including only one hot meal per day, which almost always includes vegetables. This relative lack of contrast and thus a relatively large measurement error may result in attenuation of the estimated RR for the association of total vegetable consumption and RCC. Due to individual preferences, contrast in consumption frequency of specific vegetables is much higher, which means that attenuation is probably less important for RRs estimated for specific vegetables. It was not possible to assess validity for specific vegetables in the NLCS validation study, since 9 days of dietary record (3-day diaries completed at three time points during a calendar year) are not sufficient to estimate consumption frequency of specific vegetables. To minimize the amount of uninformative data in addition to the general dietary exclusion criteria, we excluded subjects who appeared not to have understood how to fill out the questions on vegetable consumption, which occurred in the first part of the food-frequency questionnaire; an extreme score on the vegetable error index defined those subjects. For the same cohort, statistically significant inverse associations have been described between most categories of vegetable or fruit consumption and lung cancer[24] as well as between brassica vegetables and cooked leafy vegetables and colon cancer[31], indicating that measurement error or too little contrast most likely do not mask a possible association.

To our knowledge, only three cohort studies[11,14,16] have evaluated the relationship between vegetable and/or fruit consumption and RCC. The Iowa Women's Health Study with data on 124 women reported no association for "total fruit and vegetables" and for "cruciferous or green leafy vegetables"[16]. In an earlier report from this study on 62 cases, a null association was reported for consumption of "fruit + vegetables", "fruit" and "vegetables" and RCC risk[32]. These results were confirmed in our study.

The Swedish Mammography Cohort included 122 Swedish women and observed a non-significant inverse association between the combined consumption of total fruits and total vegetables and RCC risk. Individual vegetables were associated with non-significantly decreased risks, except for root vegetables, which were statistically significantly inversely associated. For individual fruits, a non-significant inverse association with apples, a null association with citrus fruit, a statistically significant inverse association with banana and a non-significant increased risk with fruit juice consumption were observed[11]. The only agreement with the current study was the protective effect for banana consumption. A direct relationship between total phenolic content and total antioxidant activity in phytochemical extracts of different fruits has been observed, bananas ranked 6th out of 11 fruits tested[33]. Bananas had the highest

bound-W phenolics content, but the significance of bound phytochemicals in fruits to human health is not clear[33]. The other cohort study was conducted amongst Californian Seventh-day Adventists with 8 male and 6 female incident cases[14]. Participants in this study seldom smoked (only 3% current smokers and 20% ex-smokers) and were mostly vegetarian. This study only reported on green salad and fruit consumption for which a statistically non-significant inverse association was found.

Ten case-control studies[6-10,12,13,15,17,18] have evaluated the relationship between vegetable and/or fruit consumption and RCC risk. Three studies are based on a large number of cases and population based controls. The first was conducted in Los Angeles and reported on dark green vegetables, yellow-orange vegetables, tomatoes or tomato products, citrus fruits and citrus fruit juices in relation to RCC, based on 1,204 RCC cases and an equal number of neighbourhood controls matched by sex, date of birth (within 5 years) and ethnicity[8]. Only dark green vegetables showed a significant inverse association with RCC risk. Results were not different for smokers or never smokers, for persons with a BMI greater or less than 24.4 kg/m^2 or for persons with or without a history of hypertension, which was confirmed in this study. The second study was performed in Canada among 1,279 incident cases and 5,370 controls. A significant inverse association with RCC was observed with increasing total consumption of vegetables and vegetable juices for males and females combined. Statistically significant inverse associations between total consumption of dark-green vegetables (broccoli and spinach) and cruciferous vegetables (broccoli, cabbage, cauliflower and Brussels sprouts) were observed for females only. Smoking did not modify the association between vegetable and fruit consumption and RCC[9]. The other large case-control study was a multicenter study from Australia, Denmark, Sweden and the United States of America (International renal-cell cancer study)[15] and investigated total vegetables, orange/dark green vegetables, cruciferous vegetables, allium vegetables, total fruit, citrus and apples and pears among 1,185 cases and 1,526 controls. A significantly decreased risk in quartile 4 for orange/dark green vegetables was observed, with most estimates less than, but close to one and a more pronounced statistically significant inverse association with RCC in never smokers. Four of the case-control studies, referred to earlier, were part of the International Renal Cell Cancer Study[12,13,17,18]. These studies reported a statistically significantly inverse association with RCC for fruit consumption[12,13] and null associations[16-18]. However, results from one study were not included in the aggregated article because other methods were used for dietary assessment[13], while another article included a larger case group[18], and a third article additionally reported on food intake 20 years before the interview (with similar results)[12]. Data from case-control studies from Italy, Germany and China mostly showed decreased risks with vegetable and fruit consumption[6,7,10,13]. Statistically significant decreased associations were only observed for carrot consumption[10], the highest tertile of green vegetable consumption[6], total vegetable and total fruit consumption but confined to men[7] and the highest tertile of fruit consumption[6,13]. None of the earlier mentioned studies reported on mandarin

consumption, which suggests this was either not investigated or no statistically significant association was observed.
We were able to assess the independent association with specific vegetable and fruit groups and for individual vegetables and fruits by adjusting for total vegetable and/or fruit consumption. Only one cohort study[11] and one hospital based case-control study also adjusted for total vegetable/fruit consumption[6]. All significantly reduced risks reported so far seem to be confined to a subgroup such as one specific type of vegetable or fruit or to a specific tertile quartile or quintile, which may suggest chance results due to multiple testing. In our study, statistically significant results for a quintile were also incidentally observed, but we did not observe this in multivariable adjusted analyses based on the continuous vegetable or fruit variable, or in the trend over the categories.
To our knowledge this is the largest prospective study on vegetable and fruit consumption and RCC risk currently available. Our results suggest the absence of an association between vegetable and fruit consumption and RCC risk.

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Appendix 1

Composition of botanical vegetable and fruit groups.

Group name	Vegetables or fruit represented in this group
Cooked vegetables	Endive (prepared); cauliflower (prepared); kale (prepared); mushrooms; leek (prepared); spinach (prepared); Brussels sprouts (prepared); onion (prepared); carrots (prepared); sauerkraut; beets (prepared); broad beans (prepared); cabbage (prepared); sliced beans, string beans (prepared); sweet peppers; other vegetables prepared.
Raw vegetables	Raw endive; lettuce; carrots (raw); tomatoes; other raw vegetables.
Legume	Broad beans (prepared); pulses; sliced beans, string beans (prepared).
Brassica	Cauliflower (prepared), cabbage (prepared), kale (prepared); Brussels sprouts (prepared).
Leafy vegetables, cooked	Endive (prepared); spinach (prepared).
Leafy vegetables, raw	Raw endive; lettuce; cress[a].
Allium vegetables	Leek (prepared); onions (prepared); cocktail onions (sweet-sour)[a]; garlic[b].
Carrots	Carrots (prepared); canned carrots[a].
Beets	Beets (prepared); beet-juice.
Tomatoes	Tomatoes-raw; tomato-juice.
Other cooked vegetables	Other vegetables prepared[a]
Other raw vegetables	Other raw vegetables[a]
Citrusfruit	Lemons[a], fresh lemon juice1; grapefruit, fresh grapefruit juice; mandarins; oranges, fresh orange juice.
Grapes	Grapes (blue and white).
Bananas	Bananas.
Apples/pears	Applesauce; apples, pears.
Strawberries	Strawberries.
Other fruit and fruit juices	Other fruits and fruit juices[a].

[a] Vegetable or fruit not specifically asked about in the questionnaire but entered in the open-ended question where participants could fill out other foods regularly eaten; [b] Data derived from question on supplement use.

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4

Prevalence of *von Hippel-Lindau* gene mutations in sporadic renal cell carcinoma: results from the Netherlands cohort study

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Abstract

Background

Biallelic *von Hippel-Lindau* (*VHL*) gene defects, a rate-limiting event in the carcinogenesis, occur in approximately 75% of sporadic clear-cell Renal Cell Carcinoma (RCC). We studied the *VHL* mutation status in a large population-based case group.

Methods

Cases were identified within the Netherlands cohort study on diet and cancer, which includes 120,852 men and women. After 11.3 years of follow-up, 337 incident cases with histologically confirmed epithelial cancers were identified. DNA was isolated from paraffin material collected from 51 pathology laboratories and revised by one pathologist, leaving material from 235 cases. *VHL* mutational status was assessed by SSCP followed by direct sequencing, after testing SSCP as a screening tool in a subsample.

Results

The number of mutations was significantly higher for clear-cell RCC compared to other histological types. We observed 131 mutations in 114 out of 187 patients (61%) with clear-cell RCC. The majority of mutations were truncating mutations (47%). The mean tumor size was 72.7 mm for mutated tumors compared to 65.3 mm for wildtype tumors (p=0.06). No statistically significant differences were observed for nuclear grade, TNM distribution or stage. In other histological types, we observed 8 mutations in 7 out of 48 patients (15%), 1 mutation in 1 of 6 oncocytoma, 3 mutations in 2 of 7 chromophobe RCC, 2 mutations in 2 of 30 papillary RCC, no mutations in 1 collecting duct carcinoma and 2 mutations in 2 of 4 unclassified RCC.

Conclusions

VHL mutations were detected in 61% of sporadic clear-cell RCC. *VHL* mutated and wildtype clear-cell RCC did not differ with respect to most parameters.

Background

Historically, the classification of Renal Cell Cancer (RCC) was based on morphological features. The majority of RCC are of the clear cell type (~80%); other subtypes are papillary RCC (10%), chromophobe RRC (5%), collecting-duct carcinoma (1%) and unclassified RCC (3-5%). Based on the work of numerous investigators, it became evident that RCC could be divided into genetically distinct classes: this resulted in the so-called Heidelberg classification, which partly overlaps the former pathological classification of RCC based on morphological criteria. The most prominent common genetic aberration for clear-cell (conventional) RCC is loss of 3p. Characteristic for papillary RCC is trisomy of chromosomes 3q, 7,8,12,16,17,20, and loss of the Y chromosome, and chromophobe RCC is characterized by a combination of loss of heterozygosity at chromosomes 1,2,6,10,13,17, and 21[1].

Von Hippel-Lindau (*VHL*) disease is a rare inherited disorder associated with, amongst others, an enhanced risk for clear-cell RCC[2]. The VHL gene responsible for this syndrome was identified through linkage analyses and molecular cloning and is located on chromosome 3p25. After its identification it became evident that the *VHL* gene is also involved in the development of sporadic clear-cell RCC. Together with loss of the homologous chromosome 3p allele (3p LOH), *VHL* mutations are rate-limiting events in the carcinogenesis of clear-cell RCC[3,4]. Mutations have been observed in the entire gene and usually lead to a truncated inactive protein[5]. The *VHL* gene is considered a tumor suppressor gene, involved in cell cycle regulation, regulation of hypoxia inducible genes and proper fibronectin assembly in extracellular matrix[6,7]. It was estimated that approximately 75% of all sporadic clear-cell RCC harbor biallelic *VHL* defects[8]. In approximately 19% of sporadic clear-cell RCC, methylation of the *VHL* gene promoter appeared to be involved[9]. In approximately 10%-20% of sporadic clear-cell RCC no alteration in the *VHL* alleles was detected, indicating that other genes are involved in clear-cell RCC carcinogenesis, possibly affecting the same signaling pathway as VHL.

Several risk factors for developing RCC have been identified: tobacco smoking, obesity, drugs such as phenacetin, hypertension and/or its medication, and occupational exposure to trichloroethylene, gasoline, petroleum products, asbestos, and iron processing fumes. The influence of dietary factors, such as vegetable, fruit, vitamin C, carotenoid, meat and milk product consumption, is controversial[10]. Multiple and specific types of *VHL* mutations in RCC have been associated with exposure to the industrial solvent trichloroethylene[11,12]. Consumption of vegetables and citrus fruit decreased the frequency of *VHL* mutations among smokers and consumption of citrus fruit decreased *VHL* mutation frequency for all patients[13]. These findings and investigations in animals[14] suggest that mutational patterns in the *VHL* gene may serve as an etiological imprint to factors causing renal cancer. Thus, it may be possible to improve our etiological insight in particular risk factors when a more specific endpoint

than "RCC" can be defined, e.g. based on histology and mutational status of a gene involved in tumor carcinogenesis.
We decided to determine the mutational status of the *VHL* gene of RCC cases identified within a population-based cohort of 120,852 men and women aged 55-69, which was recruited in the Netherlands to study associations between dietary habits, lifestyle and cancer occurrence. To validate whether SSCP could serve as a prescreening method, SSCP and direct sequencing was evaluated in a subset of 20 patients. In this article we report on histopathological and clinical parameters and the type and number of mutations in *VHL* observed in a large population-based sample of cases.

Methods

Study population

The RCC cases selected for this study were incident cases identified in the population of the Netherlands Cohort Study (NLCS) on diet and cancer. The NLCS has been described in detail elsewhere[15]. Briefly, this prospective study was initiated in 1986 and included 58,279 men and 62,573 women, 55 to 69 years of age, who completed a self-administered questionnaire on diet, family history of cancer, and other risk factors for cancer at baseline. The entire cohort is being monitored for cancer occurrence by annual record linkage to the Netherlands Cancer Registry and to PALGA, a nationwide network and registry of histo- and cytopathology[16]. Information on sex and family history of RCC (at baseline) was retrieved from the NLCS database, while information on age at diagnosis and tumor localization was retrieved from the Netherlands Cancer Registry. Tumor stage was classified according to the 1987 revision of the UICC-TNM classification[17], using information from the cancer registry and the pathology report. Tumor size was retrieved from the pathology report.

Tissue sample collection

Paraffin material was collected after approval by the Medical Ethical Committees of Maastricht University, PALGA and the Netherlands cancer registry. From 1986 to 1997 (11.3 years follow up) 355 kidney cancer cases (ICD-O-3: C64.9) were identified. Urothelial cell carcinomas were excluded and only histologically confirmed epithelial cancers were included (ICD-O: M8010-8119, 8140-8570), leaving 337 cases. The PALGA database was also used to identify the location of tumor tissue storage in the Dutch pathology laboratories. For 273 cases, a PALGA record with information on the location of paraffin material was available at the start of the collection of paraffin blocks. The tissue samples were distributed over 51 pathology laboratories throughout the Netherlands.

For 251/273 cases (92%) paraffin blocks were collected. Failure to retrieve material was the result of the refusal of the pathology laboratory to cooperate (3 laboratories with material for 10 cases), the unavailability of suitable material (i.e. only material from a biopsy, cytology or a metastasis was present) (8 cases), not being able to locate the paraffin block at the laboratory (3 cases), and for 1 case the reason was not recorded. Paraffin blocks of parallel normal tissue were collected if available. Collected archival tissue sample blocks were registered and coded using a unique identification number.

Revision

Genomic DNA was extracted from five 20-µm slices from each tumor and normal specimen. A flanking section was haematoxylin and eosin (HE) stained for histological purposes, e.g. grading and estimation of percentage of tumor cells. One experienced pathologist (CAHK) reviewed all HE stained slides. The RCC were classified according to the World Health Organization (WHO) classification of Tumours of 2002[18]. Nuclear grading was performed according to Fuhrman[19]. Grading was based on the most atypical focus in the paraffin block used for DNA extraction, with a dimension of at least one high power field.
Material of 16 cases was discarded after revision. The collected material was unsuitable for analysis, because it concerned a biopsy (N=2) or a metastasis (N=2) or no tumor tissue was present (N=4) or material contained less than 10% malignant cells (N=7) (tumor samples had to contain at least 10% malignant cells to decrease the possibility of ignoring mutations). Material from one case was reclassified as urothelial cell carcinoma and subsequently excluded. As a result, tumor DNA from 235 cases was available for further analysis.

DNA isolation

Genomic DNA was prepared as follows: paraffin was removed with xylene and genomic DNA was extracted by salt-precipitation. Briefly, 450 µl of cell lysis solution (10 mM Tris/HCl (pH 7.4), 400 mM NaCl, 2 mM EDTA), 25 µl of 10% SDS and 50 µl of proteinase K solution (20 mg/ml) were added to the tissue samples and incubated over-night at 55°C. Proteins were precipitated using 175 µl of saturated NaCl, followed by centrifugation (2 minutes, 13.200 rpm). DNA was precipitated by the addition of 0.6 volumes of iso-propanol, dissolved in TE (pH 7.4) and stored at -20°C. The DNA concentration and purity was measured at 260 and 280 nm.

VHL mutation analysis

PCR primers used for amplification are described in Table 4.1. Amplification of DNA for SSCP analysis and sequence analysis was performed as follows: 100 ng of the extracted DNA was subjected to 35 cycles of PCR: 40 sec at 92°C, 40 sec at Tm and 40 sec at 72°C. Exon amplification was performed in 30 µl of buffer: 50 mM KCl, 10 mM

Tris-HCl (pH 9.0), 1.5 mM MgCl2, 1% triton X-100, 0.1% (w/v) gelatin, 250 uM dNTP's, 25 pmol of each primer and 1U Taq polymerase (HT Biotechnology Ltd.). For SSCP analysis, 0.2 µl of [α32P] dATP (10 mCi/ml, ~3000 Ci/mmol, FIRMA) was added.

For SSCP analyses, 5 µl of the radiolabeled PCR product was diluted in 5 µl loading buffer (96% formamide, 20 mM EDTA, 0.05% bromophenol blue and xylene cyanol), boiled for 3 min, and quenched on ice before loading. Three µl of each sample was loaded on a 0.5x MDE gel, containing 0.6x TBE either with or without 10% (v/v) glycerol. Electrophoresis was performed at room temperature at 5W for MDE gels without glycerol, and at 7W for MDE gels with 10% (v/v) glycerol, using 0.6xTBE as electrophoresis buffer. After 20 hours of electrophoresis, the gels were transferred to Whatmann 3MM paper (FIRMA) and dried on a gel dryer. The separated fragments were visualized by Hyperfilm-MP (FIRMA) exposure.

For sequence analysis, the PCR products were purified using the Wizard™ PCR preps purification system (Promega Corp.). Sequencing was performed at the local central sequence facility using BigDye Terminator and the ABI basecaller (Applied Biosystems). Mutations were identified by visual inspection of sequences provided by the ABI basecaller, and called when unequivocally present on the sense and/or antisense strand, and when the area under the curve of peaks changed by more than 5%, compared to the area under the curve of the normal signal.

Table 4.1 Primers used to amplify the *VHL* gene.

Name	Sequence 5'>3'	Exon	Tm	Fragment length
Sense3a	GGT CTG GAT CGC GGA GGG A	1	64°C[a]	191 bp
Asense4a	GCC CGG CCT CCA TCT CCT	1		
Sense5a	AGT CGG GCG CCG AGG AGT	1	64°C[a]	184 bp
Asense6a	CCG TCG AAG TTG AGC CAT AC	1		
Sense7a	CCC AGG TCA TCT TCT GCA AT	1	64°C[b]	159 bp
Asense8a	CTG CTG GGT CGG GCC TAA G	1		
Sense9	GTG GCT CTT TAA CAA CCT TTG C	2	60°C[c]	194 bp
Asense10	CCT GTA CTT ACC ACA ACA ACC TTA TC	2		
Sense11a	CAC TGA GGA TTT GGT TTT TGC	3	55°C	162 bp
Asense12a	TCC AGG TCT TTC TGC ACA TTT	3		
Sense13a	GAC ATC GTC AGG TCG CTC TA	3	55°C	150 bp
Asense14a	TCA AAA GCT GAG ATG AAA CAG TG	3		

[a] 3% DMSO added for PCR; [b] 1.5% DMSO and 2.5 mM $MgCl_2$ added for PCR; [c] 4% DMSO and 1.0 mM $MgCl_2$ added for PCR

Feasibility and comparability pilot

Twenty samples were analyzed by PCR-SSCP and direct sequencing to investigate the suitability of PCR-SSCP as a screening tool preceding direct sequencing. After evaluation, we decided to use PCR-SSCP as a screening instrument before direct

sequencing. All remaining samples were subjected to PCR-SSCP, which was only followed by direct sequencing in case of an aberrant band pattern on the PCR-SSCP. Samples were also sequenced when equivocal PCR-SSCP results were obtained.

Statistical analyses

The overall frequency of *VHL* mutations as well as the type of mutation and affected exon and codon were computed for all 235 cases. Differences between histological subtypes were assessed by the χ^2-test. For clear-cell RCC, mutated and wildtype tumors were compared with respect to age at diagnosis, sex, grade, TNM classification[17], stage and family history of RCC. Differences in mean values of age at diagnosis as a continuous variable were evaluated by the Student t-test. Differences in the categorical variables sex, family history of RCC, grade, TNM classification and stage were evaluated for significance by the χ^2-test. A p value of 0.05 or less was considered statistically significant. Statistical analyses were performed with the STATA statistical software package (STATA statistical software, Release 7, STATA corporation, College Station, TX, USA, 2001).

Results

PCR-SSCP as a screening tool preceding direct sequencing

To evaluate whether PCR-SSCP could be used to distinguish between wildtype and mutated *VHL*, we determined the *VHL* status of 20 cases by SSCP and direct sequencing (Table 4.2). SSCP and direct sequencing were mostly in agreement. In all cases where disparate results were obtained, this concerned aberrant signals in PCR-SSCP, followed by a negative result by direct sequencing (Table 4.2). Most importantly, we never found a mutation in the sequence analysis after a negative PCR-SSCP (0% false-negatives) (Table 4.2). However, we are aware that the sampling size is limited, and it is known that SSCP as a screening tool is neither 100% sensitive nor specific. Therefore, we estimated the chance of a positive result on direct sequencing after a negative result on the SSCP (i.e., the chance that a mutation will be missed by using SSCP as a screening instrument). The point estimate for the percentage of false-negatives based on SSCP is 0%, with an upper confidence limit between 3.7% and 16.9%, based either on the individual analyses (N=97), or on the number of cases (N=20) (see Additional file 1: Calculation of the estimated upper 95% confidence limit). Different types of mutations were identified by SSCP followed by direct sequencing in this pilot.

Table 4.2 Mutation analysis in 20 samples by SSCP and direct sequencing.

			Direct sequencing	
			Positive	Negative
Primerset 3/4	SSCP	Positive	0	5
		Negative	0	15
Primerset 5/6	SSCP	Positive	2	0
		Negative	0	18
Primerset 7/8	SSCP	Positive	4	2
		Negative	0	14
Primerset 9/10	SSCP	Positive	3	0
		Negative	0	17
Primerset 11/12	SSCP	Positive	4	1
		Negative	0	15
Primerset 13/14	SSCP	Positive	2	0
		Negative	0	18

VHL mutations and clinical parameters

We were able to analyze at least one tumor block for 235 patients with 236 tumors (one patient had two primary tumors). After revision by CAHK, the analyzed samples could be subdivided as follows: clear-cell RCC (188/236, 79.7%), papillary RCC (30/236, 12.7%); chromophobe RCC (7/236, 3.0%); oncocytoma (6/236, 2.5%), collecting duct carcinoma (1/236, 0.4%), and unclassified RCC (4/236, 1.7%). For 198 cases one paraffin block was analyzed, while for 34 cases 2 paraffin blocks were analyzed and for 3 cases 3 paraffin blocks were analyzed. In 10 cases, a different genotype was observed in different tumor blocks obtained from 1 tumor. In 6 cases, 1 sample contained wildtype *VHL* while the other sample harbored one or more mutations. For the statistical analyses these cases were analyzed as "mutated". In 13 cases, 2 mutations in the same paraffin block were observed. In one patient with two primary tumors, both tumors showed a different mutation. In total we observed 253 outcomes (139 mutations (in 121 patients) and 114 wildtype) for 235 patients with 236 primary tumors. All 139 observed mutations are described. (see Additional file 2: Description of observed mutations (N=139), histological parameters and personal characteristics for 121 cases). *VHL* gene mutations were mostly observed in clear-cell RCC, but also in unclassified RCC (2/4); papillary RCC (2/30); chromophobe RCC (3 mutations in 2/7 patients) and oncocytoma (1/6) (see Additional file 2: Description of observed mutations (N=139), histological parameters and personal characteristics for 121 cases). The percentage of patients with a mutation was significantly higher for clear-cell tumors compared to tumors of other histological types (χ^2: 32.9; p value<0.001). In the remainder of this paper, we will only consider clear-cell RCC, leaving 204 outcomes (131 mutations (in 114 patients) and 73 wildtype) for 187 patients with 188 primary tumors. Nine outcomes were mutations in intronic sequences, possibly affecting splicing. The majority of observed mutations in coding regions lead to a truncated VHL product

(insertion/deletion mutations leading to a frameshift and nonsense mutations, 62/122) or to a deleted/inserted or altered amino acid, 47/122). The percentage of point mutations was much higher in exon 1 (50.7%) than in exon 2 and 3 (29.6% and 23.1%, respectively).

Figure 4.1 shows the type of mutation plotted against the codon number. We observed 62 truncating mutations, which consisted of 4 nonsense mutations and 58 deletions or insertions leading to a shift of the reading frame (Figure 4.1A), 15 insertions or deletions that did not affect the reading frame (Figure 4.1B), 32 missense mutations (Figure 4.1C) and 13 silent mutations (Figure 4.1D).

Cases with a mutation were older (mean: 67.8 years; sd: 4.7) than cases without a mutation (mean: 67.1; sd: 4.6). The mean age difference at diagnosis was 0.8 years with a 95% CI ranging from –0.6 through 2.2 years. The percentage of men with clear-cell RCC with a *VHL* mutation was somewhat higher than the percentage of males among patients with a clear-cell RCC without a *VHL* mutation, 63.2% vs. 53.4% (χ^2: 1.75; *p* value: 0.19).

The mean tumor size was 72.7 mm for tumors with a *VHL* mutation compared to 65.3 mm for tumors without a *VHL* mutation. The difference was 7.3 mm with a 95% CI from –2.1 mm through 16.8 mm. Other tumor parameters for patients with a primary clear-cell RCC with or without a *VHL* mutation are shown in Table 4.3. We did not observe a difference in nuclear grade, T, N, M, or stage between mutated and wildtype tumors.

For 3 patients, a positive family history was reported; 2 of these patients (Sample id's: 1003 and 306/1600) had a clear-cell tumor harboring a mutation. One patient with a papillary tumor reported a positive family history; no mutations in the *VHL* gene were observed in the corresponding tumor sample. Obviously, these numbers are too small to engage in statistical testing.

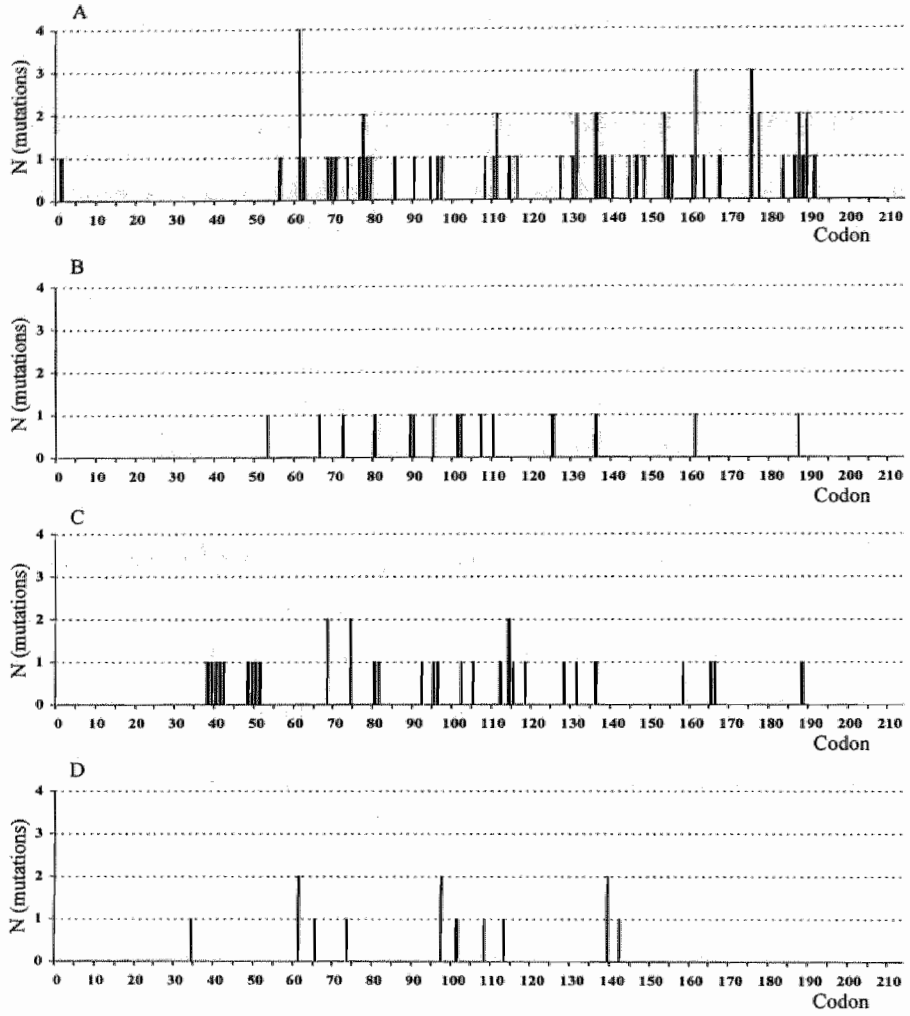

Figure 4.1 Type of mutation plotted against the codon number.

A. Truncating mutations (Frameshift & Nonsense mutations)
B. In-frame insertions/deletions
C. Missense mutations
D. Silent mutations

Codon 1-114 encodes exon 1, codon 114-155 encode exon 2, and codon 155-213 encode exon 3.

Table 4.3 Tumor parameters for patients with a clear-cell tumor, also stratified by *VHL* mutation status.

	All tumors (N=188)		VHL mutated tumors (N=115)		Wildtype tumors (N=73)			
	N	%	N	%	N	%	χ^2	*p* value[b]
T (TNM[a])								
1	5	2.7	4	3.5	1	1.4		
2	93	49.5	55	47.8	38	52.1		
3A	35	18.6	20	17.4	15	20.6		
3B	50	26.6	34	29.6	16	21.9		
4	2	1.1	1	0.9	1	1.4	0.18[d,f]	0.67
X	3	1.6	1	0.9	2	2.7		
N (TNM[a])								
0	126	67.0	78	67.8	48	65.8		
1	7	3.7	6	5.2	1	1.4		
2	7	3.7	5	4.4	2	2.7	1.51[e,f]	0.22
X	48	25.5	26	22.6	22	30.1		
M (TNM[a])								
0	131	69.7	77	67.0	54	74.0		
1	26	13.8	18	15.7	8	11.0	0.99[f]	0.32
X	31	16.5	20	17.4	11	15.1		
Stage (TNM[a])								
1	5	2.7	4	3.5	1	1.4		
2	83	44.2	46	40.0	37	50.7		
3	66	35.1	42	36.5	24	32.9		
4	32	17.0	22	19.1	10	13.7	1.41[d,f]	0.24
X	2	1.1	1	0.9	1	1.4		
Nuclear grading[b,c]								
I	46	24.5	27	23.5	19	26.0		
II	67	35.6	43	37.4	24	32.9		
III	48	25.5	29	25.2	19	26.0		
IV	27	14.4	16	13.9	11	15.1	0.43	0.94

[a] Based on pathological TNM unless unknown, then clinical TNM (UICC, 1987) was used[17]; [b] Based on the review by one experienced pathologist (CAHK); [c] According to Fuhrman[19]; [d] 1-2 vs. 3-4; [e] 1-2 vs. 0; [f] Excludes "X (unknown)" category.

Discussion

The *VHL* gene is a tumor suppressor gene predisposing to both sporadic clear-cell (conventional) RCC and von Hippel-Lindau disease. We analyzed 235 cases of primary sporadic RCC, of which 187 cases presented with the histological subtype clear-cell RCC, identified within the Netherlands cohort study on diet and cancer. *VHL* mutations were assessed by PCR-SSCP, followed by direct sequencing when aberrant signals

were detected in SSCP. Screening by SSCP was considered appropriate, since we never detected a mutation by direct sequencing after a negative result on the SSCP in a pilot study. Furthermore, we estimated the upper 95% confidence limit for cases missed at 3.7% or 16.9%, based on the individual analyses (N=97) or on the number of cases (N=20). Therefore, mutated cases may have been overlooked, which placed them in the wildtype group. This would have resulted in a reduction of contrast between wildtype and mutated groups.

We observed 131 mutations in 114 out of 187 patients (61%) in patients with clear-cell tumors. Estimates reported in the literature[2,20-30] range from 36%[25] to 54%[27], and therefore our estimate appears high. However, our estimate is close to the estimate of 75% as discussed in the review by Cohen[8]. This difference in the observed proportion of mutations in the *VHL* gene may be attributed to the use of different techniques, e.g., SSCP vs. direct sequencing, to contamination with normal tissue components, or to the type of material analyzed (fresh or archival paraffin material). In our study, we used SSCP followed by direct sequencing, as most investigations did[2,20-29]. To avoid ignoring *VHL* mutations through contamination with normal tissue, samples needed to contain at least 10% tumor cells. The vast majority of samples (180 out of 190) contained at least 50% viable tumor tissue. The sample with the lowest percentage of viable tumor tissue, in which we observed a mutation, contained 30% of viable tumor tissue. For 2 samples the percentage of viable tumor tissue was lower (10% and 20%), thus we cannot formally exclude the possibility that we overlooked an existing mutation. We identified cases in a population-based study after 11.3 years of follow-up; the time span and the number of patients and hospitals involved excluded the possibility to collect fresh material. Three other studies on sporadic RCC used paraffin embedded material and the percentages of mutations detected were 46%[29], 50%[22] and 54%[27].

The occurrence of various types of mutations is comparable to other studies of this size[21,24,26,27], but some differences are also apparent. The presence of silent mutations is not commonly reported; only Ma *et al.*[27] reported 13% of silent mutations, similar to 11% observed by us. It is possible that others do not report silent mutations because the investigators do not wish to designate these as true mutations since their relevance is unclear. The percentage of frameshift mutations was approximately 50% in most studies[21,26], comparable to the 48% observed in the current study. The percentage of in-frame deletions/insertions was higher in our study (12%) as compared to the percentage in other studies (<5%)[21,26]. Compared to the distribution of mutation data present in the Universal *VHL* Mutation database with 747 mutations[31], the relative amount of point mutations is much higher in the database (64%), compared to the percentage of point mutations we observed (44%), as well as the frequency of nonsense mutations (11% vs. 3%). However, many of the mutations in the Mutation database concern germline mutations identified in VHL-families. It has been suggested that the mutational spectrum between germline and sporadic *VHL* mutations may differ[25], which could explain the disparity. We were not able to select records for sporadic tumors in the

database, but Gallou *et al.*[26] described mutations for 145 sporadic cases from this database. Compared to our results, the percentage of nonsense mutations was higher (8% vs. 3%) and the percentage of in-frame deletions or insertions was lower (4% vs. 12%)[26].

Furthermore, we searched the Universal *VHL* Mutation database[31] for the mutations observed in the cases with a positive family history of RCC. The T>A (silent) mutation at codon 139 (sample id 1003) was not reported before, the deletion of 1 nucleotide at codon 146 (sample id 306) was recorded in the database twice, while the second 1 nucleotide deletion at codon 167 (sample id 1600) had not been reported before.

For 10 RCC cases, we observed different *VHL* genotypes in different tumor samples obtained from one tumor. Samples were re-analyzed to exclude technical errors. Again, different *VHL* genotypes, identical to the earlier genotypes, were observed, showing that tumor heterogeneity appears to play a role. This was an unexpected finding in view of the general acceptance that a mutation in the *VHL* gene is an early event in clear-cell RCC carcinogenesis, because one would then expect monogenetic *VHL* expression. Nevertheless, it is possible that genetic instability leads to additional mutational events, eventually leading to different *VHL* genotypes in clear-cell RCC. This was underscored by 13 cases showing 2 mutations within the same paraffin block and other studies have also reported multiple mutations per primary tumor[21,27,29].

The distribution of histological subtypes was comparable to the distribution described before[32,p2692]. Surprisingly, we also found *VHL* mutations in some of the non-clear-cell RCC samples that were collected. These results are in contrast with the current opinion that *VHL* mutations are exclusively restricted to clear-cell RCC. *VHL* mutations, however, have also been reported in chromophobe RCC, although these were clustered in the 5'UTR/promoter region[22], and Brauch *et al.*[12] described a renal oncocytoma with a *VHL* mutation. To our knowledge, others have not yet described *VHL* mutations in papillary RCC. It is conceivable that genetic instability following tumor-initiating events leads to *VHL* mutations acquired at a later stage of tumor development. As expected, the relative occurrence of *VHL* mutations in the non-clear-cell RCC renal tumors was much lower than in clear-cell RCC, emphasizing the crucial role of *VHL* mutations in clear-cell RCC carcinogenesis.

The high percentage of mutations observed, the observation of mutational heterogeneity (different genotypes in 1 or different samples from the same tumor), and the observation of mutations in other histological types, raises the question whether paraffin material is suitable for analysis. It has been shown, however, that *VHL* gene mutations can be detected in archival paraffin material[33]. Identical mutations were observed in exon 2 in 3 cases for which archival paraffin material and tumor derived cell lines were assessed by SSCP and sequencing[33]. On the other hand, it has also been reported that artifacts may occur when archival paraffin material is used[34]. Williams *et al.* reported up to 1 mutation artifact per 500 bases recorded. The chance of such artificial mutations in formalin-fixed material was inversely correlated with the number of cells used in the PCR: the fewer the cells, the more artifacts. No artifacts were

observed when 300 cells were used and only 1 artifact was observed in 150 cells (0.03%). The highest frequency of artifacts was observed when using 10 or 20 cells, and equaled 0.2%[34]. The number of cells in our analyses, however, was large (>500 cells), since 5 20-µm slices of paraffin embedded material were used and biopsies were excluded.

The mean tumor size was larger for mutated tumors than for wildtype tumors, however this was not statistically significant. Two other studies did not show a difference in tumor size[24,28]. *VHL* gene mutations leading to reduced or inactive VHL protein (pVHL) could theoretically lead to a larger tumor size. In the absence of pVHL, hypoxia inducible factor becomes stabilized and upregulates a myriad of hypoxia-inducible genes, resulting in hypervascular tumors[35]. This should be a growth advantage. Our results and results from others[24,28] showed no association with nuclear grade (according to Fuhrman)[19]. It was difficult to compare TNM and stage, since different classifications were used in different studies and the TNM classification has changed definitions in recent years. We reviewed all cases and classified them according to the 1987 version of the TNM classification[17]. We did not observe a difference in T, N, M, or stage between mutated and wildtype tumors, as was confirmed by 2 other studies[24,28]. One study, however, reported a higher percentage of mutations in pT3 tumors with a *VHL* mutation or hypermethylation, compared to tumors without *VHL* alteration (64% vs. 40%)[21].

Phenotype should also be considered. Mutations may impair pVHL function. However, in order for pVHL to lose its function, biallelic *VHL* defects have to occur. We did not measure LOH, since it is well documented that LOH commonly occurs in RCC cases, with estimates ranging from 74%[30] to 93%[21]. Kondo *et al.* showed 97% LOH in mutated or hypermethylated samples compared to 81% LOH in wildtype samples[24]. Furthermore, silent mutations do not lead to an altered protein, but these types of mutations were not common (13/131) in our study. Also, pVHL formation may be inhibited by epigenetic silencing, which has been reported to occur in 19% of clear-cell RCC[9]. This could have resulted in misclassification in comparing pathological parameters, because samples with epigenetic silencing only were classified as wildtype tumors, as we were not able to investigate epigenetic silencing in the current study. Finally, cells in cell culture have been shown to produce two types of proteins: pVHL30 and pVHL19 (a results of internal translation from the second methionine within the *VHL* open reading frame (Met 54)[36-38]. The pVHL19 product has been shown to inhibit the production of hypoxia-inducible genes, but does not bind to fibronectin[36,37]. Reintroduction of pVHL19 has been shown to inhibit tumor formation in nude mice[37]. We detected mutations 5' of codon 54 in 11 cases; these are unusual and would be predicted to affect pVHL30, but not pVHL19 translation products. However in 5 of these 11 cases, a second mutation was observed 3'of codon 54. So, in 5 of these cases the other mutation may be relevant. For the other 6 cases, these mutations may not lead to a functionally inactive protein, and thus we may have

misclassified these. However, as remarked before, the pVHL19 variant does not bind to fibronectin and thus may not contribute to the maintenance of the extracellular matrix.

Conclusions

VHL mutations were observed in 61% of sporadic clear-cell RCC. No differences were observed in nuclear grade, TNM distribution or stage between *VHL* mutated and wildtype clear-cell RCC. However, the tumor size was larger for *VHL* mutated clear-cell RCC. This study forms a good basis to study possible associations between potential risk factors and *VHL* mutations in clear-cell RCC. Ultimately, this should lead to greater insight in the etiology of clear-cell RCC.

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Additional file 1

Calculation of the estimated upper 95% confidence limit

The point estimate for the percentage of samples negative on the SSCP and positive on direct sequencing equals 0% (Table 4.2); the upper confidence limit was calculated by the following formula: $P(X=0 \mid N, Pu) = 0.025 \rightarrow (1-Pu)^N = 0.025$. This analysis was performed for two situations. Situation 1; calculation of upper 95% confidence limit based on the cases and situation 2; calculation of upper 95% confidence limit based on the individual analyses. The N differs for these situations described; for situation 1, N equals 20 (there are 20 cases for with at least one negative SSCP, followed by a negative result on direct sequencing), for situation 2 N equals 97 (of all 120 SSCP results, 97 were negative and were followed by a negative result on direct sequencing). The estimations of the upper confidence limit are: based on situation 1: $P(x=0|N=20, P=Pu)=0.025 \rightarrow (1-Pu)^{20}=0.025 \rightarrow Pu=0.1685$ and based on situation 2: $P(x=0|N=97, P=Pu)=0.025 \rightarrow (1-Pu)^{97}=0.025 \rightarrow Pu=0.0373$. However, both of these estimates are not completely true, because in the first situation we assume all the analyses on all primer sets to be dependent and for the second estimation, we assume all the analyses of all primer sets to be completely independent, neither of which is true. Primer sets are not completely independent, since some primer sets were selected to overlap to sequence the whole coding sequence of the gene. In total, 204 out of 1042 nucleotides were determined by more than 1 primer set. Also, the quality of the extracted DNA and other properties of the DNA may render the analyses by different primer sets not independent.

Additional file 2

Description of observed mutations (N=139), histological parameters and personal characteristics for 121 cases.

Sample	Sex / Age at incidence	TNM classification[a]			Stage[a]	Histology[b]	Nuclear Grade[b,c]	% Vital tumor tissue[b]	*VHL* status Mutation[d]
		T	N	M					
941	M / 71	2	0	0	II	Clear-cell	I	75	[c.1-1_20del21 (+) c.291_310del20]
349	F / 68	2	0	0	II	Clear-cell	III	50	c.102C>T
2,048	M / 65	3B	0	0	III	Clear-cell	IV	85	c.113C>T
1,782	M / 66	3B	2	X	IV	Clear-cell	III	90	[c.115G>A (+) c.291C>A]
1,716	F / 58	2	X	X	II	Clear-cell	II	50	[c.118C>T (+) c.145G>T]
973	M / 61	3B	0	0	III	Clear-cell	III	60	[c.122A>T (+)
974							III	90	c.183C>T]
1,013	F / 70	2	1	1	IV	Clear-cell	III	100	c.125A>T
187	M / 74	3B	0	0	III	Clear-cell	II	100	c.142C>G
336	M / 65	2	0	0	II	Clear-cell	II	90	[c.149C>G (+) c.221T>C]
1,524	M / 64	2	0	X	II	Clear-cell	II	100	[c.151G>C (+)
1,525							II	100	c.284C>G]
463	F / 66	3B	0	0	III	Clear-cell	III	100	c.158ins45
1,510	F / 68	2	0	0	II	Clear-cell	I	100	c.166del1
2,109	F / 65	2	0	0	II	Clear-cell	I	100	[c. 183del1 (+) c. 463+5T>A]
191	F / 66	2	0	0	II	Clear-cell	I	90	c.183_187del5
683	M / 74	3A	0	0	III	Clear-cell	II	100	c.183_187del5
1,306	M / 62	3A	0	0	III	Clear-cell	II	100	c.183C>T
260	M / 69	2	0	0	II	Clear-cell	I	100	c.183ins5
14	M / 66	2	0	0	II	Clear-cell	II	100	c.185ins5
669	M / 65	3B	0	0	III	Clear-cell	IV	70	c.195G>C
829	M / 66	3A	0	0	III	Clear-cell	III	60	c.198_221del24
592	M / 73	2	0	0	II	Clear-cell	III	100	[c.202T>A (+) c.203C>A]
1,809	F / 65	2	0	0	II	Clear-cell	II	90	[c.204del1 (+) c.283_297del15]
2,002	F / 65	3B	0	0	III	Clear-cell	III	90	c.205del1
757	M / 67	3B	1	X	III	Clear-cell	I	95	c.208G>T
2,043	M / 70	3A	0	0	III	Clear-cell	III	70	c.215_259del45
2,438	M / 77	3B	0	1	IV	Clear-cell	II	50	c.217del1
2,035	M / 72	2	0	1	IV	Clear-cell	I	90	c.219G>A
2,049	M / 68	3A	X	X	III	Clear-cell	IV	90	c.221T>A
597	F / 71	2	0	0	II	Clear-cell	I	95	c.227del1

►

Sample	Sex / Age at incidence	TNM classification[a]			Stage[a]	Histology[b]	Nuclear Grade[b,c]	% Vital tumor tissue[b]	*VHL* status Mutation[d]
		T	N	M					
1,323	M / 58	4	X	1	IV	Clear-cell	IV	100	c.231_232del2
593	M / 70	2	0	0	II	Clear-cell	II	95	c.231C>A
334	M / 70	3B	X	0	III	Clear-cell	III	80	c.234_237del4
562	M / 65	3B	0	0	III	Clear-cell	II	100	c.236_237del2
1,798	M / 67	2	X	X	II	Clear-cell	III	95	c.239_241del3
926	M / 73	3B	1	0	III	Clear-cell	II	70	c.240T>A
949	M / 60	3B	2	X	IV	Clear-cell	III	50	c.241C>T
879	M / 70	2	0	X	II	Clear-cell	III	48	c.253ins23
1,502	M / 65	2	0	1	IV	Clear-cell	III	100	c.268_270del3
1,651	F / 58	2	0	0	II	Clear-cell	III	100	c.268_274del7
6	M / 64	2	0	0	II	Clear-cell	II	100	c.270_272del3
7	M / 64	3A	1	1	IV	Clear-cell	III	50	c.274G>T
424	F / 69	2	0	X	II	Clear-cell	I	100	c.282ins1
229	M / 75	2	0	0	II	Clear-cell	IV	45	c.288_291del4
2,088	F / 70	2	0	0	II	Clear-cell	II	67	c.288G>T
1,339	F / 68	3B	0	0	III	Clear-cell	III	50	c.291C>T
755	M / 67	3B	X	0	III	Clear-cell	II	95	c.301C>T
1,342	F / 74	2	0	0	II	Clear-cell	II	90	c.302_304del3
176	M / 69	3B	X	X	III	Clear-cell	II	100	[c.304C>G (+) c.444del1]
695	F / 69	3B	0	0	III	Clear-cell	II	90	c.306_308del3
343	F / 60	2	0	0	II	Clear-cell	III	70	c.314C>T
954	M / 70	3A	X	X	III	Clear-cell	IV	100	[c.321_323del3 (+) c.426T>G]
161	M / 69	2	0	0	II	Clear-cell	I	66	c.324C>A
1,350	F / 65	1	0	0	I	Clear-cell	I	85	c.324del1
965	M / 67	3B	X	X	III	Clear-cell	I	90	c.328_333del6
49	F / 66	3B	X	1	IV	Clear-cell	II	90	[c.330del1 (+) c.381del1]
307	M / 63	2	X	X	II	Clear-cell	II	95	c.331del1
48	M / 63	3B	0	0	III	Clear-cell	IV	100	c.333del1
189	M / 58	3B	0	0	III	Clear-cell	III	95	c.334T>G
1,912	M / 66	3A	0	0	III	Clear-cell	III	100	c.339A>T
904	F / 65	2	1	0	III	Clear-cell	II	100	c.340G>T
38	F / 66	3A	0	0	III	Clear-cell	II	100	[c. 340G>A (+) c. 340+2T>G]
2,458	F / 57	3A	X	0	III	Clear-cell	I	85	c.340+9C>T
262	F / 64	2	0	0	II	Clear-cell	I	90	c.341-2del1
690	F / 75	3B	X	1	IV	Clear-cell	III	60	c.341-1_367del28
1,299	M / 72	2	0	0	II	Clear-cell	II	90	c.343C>A
596	F / 66	2	0	0	II	Clear-cell	III	100	c.348_354del7
338	M / 68	3B	0	0	III	Clear-cell	III	100	c.353T>A

▶

Sample	Sex / Age at incidence	TNM classification[a]			Stage[a]	Histology[b]	Nuclear Grade[b,c]	% Vital tumor tissue[b]	*VHL* status Mutation[d]
		T	N	M					
9	M / 70	3A	X	1	IV	Clear-cell	IV	100	c.373_378del6
400	F / 73	2	0	X	II	Clear-cell	II	90	c.383T>A
2,057	M / 68	2	X	X	II	Clear-cell	IV	50	c.390del1
2,451	F / 66	2	0	1	IV	Clear-cell	II	95	c.391A>T
1,167	M / 74	1	0	0	I	Clear-cell	I	30	c.393_396del4
2,446	M / 72	3B	1	X	III	Clear-cell	III	85	c.393_396del4
559	M / 67	3A	0	0	III	Clear-cell	I	100	c.406_408del3
854	M / 65	3A	0	0	III	Clear-cell	III	70	c.406ins1
2,078	M / 71	3A	0	1	IV	Clear-cell	III	80	c.408del1
777	M / 67	3B	0	0	III	Clear-cell	II	100	c.408T>G
309	M / 63	3A	0	X	III	Clear-cell	I	100	c.409del1
2,449	F / 75	2	0	0	II	Clear-cell	II	100	c.413_414del2
947	M / 80	X	0	0	X	Clear-cell	II	100	c.417T>A
1,003	F / 70	1	0	0	I	Clear-cell	I	60	c.417T>A
670	M / 77	3B	2	0	IV	Clear-cell	I	60	c.418_425del8
461	F / 65	3A	0	0	III	Clear-cell	I	100	c.431del1
306	M / 67	3B	2	0	IV	Clear-cell	IV	75	[c.437del1 (+)
1,600							IV	75	c.501del1]
651	F / 71	3A	X	1	IV	Clear-cell	IV	70	c.457_463del7
1,334	F / 75	2	0	1	IV	Clear-cell	II	100	c.458ins4
952	M / 72	2	0	1	IV	Clear-cell	IV	100	c.462ins1
398	F / 70	2	X	0	II	Clear-cell	IV	100	c.463+3A>T
2,092	F / 62	3B	2	1	IV	Clear-cell	IV	80	c.463+8C>T
989	M / 66	2	0	0	II	Clear-cell	I	90	c.463+23A>G
1,325	M / 69	2	X	0	II	Clear-cell	I	85	c.464-2_469del8
2,455	M / 71	2	0	0	II	Clear-cell	II	70	c.464-1G>A
1,332	M / 66	2	0	X	II	Clear-cell	II	90	c.464-1G>C
1,303	M / 66	2	0	0	II	Clear-cell	II	70	c.472C>G
682	M / 75	2	X	0	II	Clear-cell	III	95	[c.480del1 (+) c.482_483del2]
170	F / 67	2	0	0	II	Clear-cell	II	67	c.481_483del3
692	F / 70	2	X	0	II	Clear-cell	I	85	c.481C>T
2,127	F / 74	3B	0	X	III	Clear-cell	IV	90	c.482ins1
960	M / 60	2	0	0	II	Clear-cell	II	90	c.487del1
1,329	M / 73	2	0	1	IV	Clear-cell	II	95	c.494T>A
944	M / 69	2	0	0	II	Clear-cell	III	100	c.497T>G
568	F / 76	3A	X	0	III	Clear-cell	I	50	c.523del1
1,314	M / 66	3B	0	0	III	Clear-cell	II	50	c.525_532del8
1,170	M / 66	1	0	0	I	Clear-cell	II	80	c.525C>A
1,297	M / 59	3A	X	1	IV	Clear-cell	IV	100	c.529del1
787	M / 70	3A	0	0	III	Clear-cell	III	50	c.529ins1
11	M / 69	2	X	0	II	Clear-cell	II	100	c.547del1

▶

Sample	Sex / Age at incidence	TNM classification[a]			Stage[a]	Histology[b]	Nuclear Grade[b,c]	% Vital tumor tissue[b]	*VHL* status Mutation[d]
		T	N	M					
506	F / 76	2	0	0	II	Clear-cell	II	80	[c.559_560del2 (+) c.563del1]
496	M / 66	3B	0	0	III	Clear-cell	I	100	[c.559_560del2 (+)
497		(2)					II	100	c.561_563del3]
1,001	F / 61	2	X	X	II	Clear-cell	II	100	c.561_564del4
1	M / 60	3B	0	0	III	Clear-cell	II	100	c.563T>A
369	M / 74	2	X	1	IV	Clear-cell	I	80	c.566_569del4
1,004	F / 69	2	0	0	II	Clear-cell	I	100	c.567del1
2,086	F / 74	3B	X	0	III	Clear-cell	II	100	c.573_577del5
962	M / 74	3B	0	0	III	Chromophobe	IV	50	[c.1_17del17 (+)
963							IV	50	c.471T>A]
347	M / 67	3A	0	0	III	Chromophobe	II	40	c.333C>T
1,016	F / 65	2	X	X	II	Oncocytoma	X	90	c.113C>T
1,499	M / 69	2	0	0	II	Papillary	II	100	c.264G>C
31	M / 63	2	0	X	II	Papillary	III	100	c.443T>C
2,456	F / 73	2	3	0	IV	Unclassified	IV	80	c.245G>C
16	F / 65	3A	0	0	III	Unclassified	IV	90	c.353T>C

[a] Based on pathological TNM unless unknown, then clinical TNM (UICC, 1987) was used[17]; [b] Based on the review by one experienced pathologist (CAHK); [c] According to Fuhrman[19]; [d] The notation of *VHL* gene mutations was based on the guidelines described at the following website: www.genomic.unimelb.edu.au/mdi/mutnomen/examplesDNA.html (Exon 1: c.1 through c.340; exon 2: c.341 through c.463; exon 3: c464 through c.642)

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5

Cigarette smoking, *von Hippel-Lindau* gene mutations and sporadic renal cell carcinoma

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Abstract

Cigarette smoking is a known risk factor for renal cell carcinoma (RCC) with a modestly increased rate ratio (RR) and a positive dose-response relation. *Von Hippel-Lindau (VHL)* gene mutations are considered a primary event in the carcinogenesis of RCC. We investigated whether smoking is associated with mutations in the *VHL* gene in sporadic RCC.

The Netherlands Cohort Study on diet and cancer (NLCS) includes 120,852 persons, who completed a self-administered questionnaire on smoking habits and other factors. After 11.3 years of follow-up, 337 cases and a random sample of 5,000 persons (subcohort) were used in a case-cohort approach. Collected tumor DNA (N=235) was analyzed for *VHL* gene mutations. RRs and corresponding 95% confidence intervals (CI) were estimated using Cox proportional hazard models, adjusting for age, sex and body mass index.

For men, RRs for total RCC were 1.52 (95% CI: 0.89-2.59) and 2.07 (95% CI: 1.20-3.56) for ex- and current smokers compared to never smokers, respectively. Estimates for women equaled 0.95 (95% CI: 0.57-1.59) and 1.37 (95% CI: 0.87-2.16), respectively. For men, RRs for current smokers compared to never smokers were 2.34 (95% CI: 0.79-6.94) and 2.95 (95% CI:0.65-13.28) for *VHL* gene mutated and *VHL* wildtype tumors, respectively. For women, these estimates were 0.82 (95% CI:0.35-1.93) and 2.04 (95% CI:0.94-4.45), respectively. Results have to be interpreted cautiously because of small numbers.

Smoking was associated with RCC risk of men, but not specifically with *VHL* gene mutations, irrespective of sex, suggesting that smoking may cause RCC independent of *VHL* gene mutations.

Introduction

Renal Cell Carcinoma (RCC) is the ninth most common tumor in the European Union[1] and is associated with a 5- year survival of approximately 55%[2]. Both the incidence and mortality rates are about twice as high for males than females[1].
Mutations in the *von Hippel-Lindau (VHL)* gene are perceived to be an early event in renal carcinogenesis and are mainly observed in tumors of the most common histological subtype, clear-cell RCC[3]. Mutations are observed in the entire gene and usually lead to a truncated inactive protein[3]. The *VHL* gene is a tumor suppressor gene involved in cell cycle regulation, regulation of hypoxia inducible genes and proper fibronectin assembly in extracellular matrix[4,5]. It is estimated that 56 to 69%[4] of clear-cell renal tumors harbor a mutation in the *VHL* gene.
Occupational exposure to trichloroethylene[6,7] and consumption of citrus fruit and vegetables (confined to smokers)[8] have been linked to *VHL* gene mutations in renal cell carcinoma (RCC) in previous studies[6-8]. There was no difference in the proportion of smokers for cases with or without a *VHL* gene mutation, but in this case-only study by Hemminki *et al.* no comparison to the population could be made[8]. To our knowledge, there are no other reports on an association of smoking and *VHL* gene mutations published thus far.
A recent meta-analysis on cigarette smoking in relation to renal cell carcinoma (RCC) concluded that inhaled tobacco smoke is clearly implicated in the etiology of RCC. Risk was increased for former (RR: 1.21; 95% CI: 1.07-1.36) and current (RR: 1.45; 1.26-1.66) smokers with a strong dose-dependent increase in risk[9]. It has also been shown that cigarette smoke metabolites can cause mutations in human DNA, which is not restricted to tissues directly exposed to tobacco smoke[10]. Moreover, it has been reported that specific constituents can cause specific mutations[10-12]. Also, cigarette smoke metabolites have been shown in urine[13].
We investigated whether cigarette smoking is associated with sporadic RCC and with mutations in the *VHL* gene in clear-cell RCC in a prospective cohort study.

Materials and methods

Subjects

The Netherlands Cohort Study on diet and cancer is a prospective cohort study, which started in September 1986. The study design has been reported in detail elsewhere[14]. Briefly, the cohort included 120,852 men and women, aged 55-69 years, at the beginning of the study. The study was designed as a case-cohort study, using all cases and a random sample of 5,000 persons from the cohort (subcohort), who have been followed to estimate the accumulated person-years in the entire cohort[15]. Follow-up for incident cancer has been established by computerized record linkage with the

Netherlands Cancer Registry and PALGA, a national database of pathology reports. The method of record linkage to obtain information on cancer incidence has been described previously[16]. The completeness of cancer follow-up was estimated to be more than 96 percent[17]. From 1986 to 1997 (11.3 years follow up) 355 kidney cancer cases (ICD-O-3: C64.9) were identified. Urothelial cell carcinomas were excluded and only histologically confirmed epithelial cancers were included (ICD-O: M8010-8119, 8140-8570), leaving 337 cases. All participants who reported prevalent cancer (excluding skin cancer) at baseline were excluded from analyses (leaving 4,774 subcohort members).

VHL gene mutation analysis

Paraffin material of cancer cases was collected after approval by the Medical Ethical Committees of Maastricht University, PALGA and the Netherlands cancer registry. We were able to collect paraffin blocks of tumors for 251 cases from 51 pathology laboratories, which we described in detail elsewhere[18]. One experienced pathologist (CAHK) revised all HE-stained slides. The RCC were classified according to the World Health Organization (WHO) classification of Tumours of 2002[19]. DNA isolation and mutation analyses have been described previously[18]. Briefly, paraffin was removed with xylene and DNA was extracted by salt-precipitation. The entire gene was amplified using 6 primer sets. Samples were first subjected to PCR-SSCP analysis, which was followed by direct sequencing in case of aberrant or equivocal results. Mutations were identified by visual inspection of sequences provided by the ABI basecaller. After revision and *VHL* gene mutation analyses, data was available for 235 cases[18].

Questionnaire

At baseline, all cohort members completed a mailed, self-administered questionnaire on dietary habits (food-frequency questionnaire), lifestyle, smoking, personal and family history of cancer and demographic data[20]. Questions on cigarette, cigar and pipe smoking in the questionnaire addressed smoking status (never, ex or current smoker), age at first and last exposure, frequency, duration and inhalation pattern. Brand and type of cigarette and how much of a cigarette was usually smoked was additionally questioned, as well as use of chewing and snuff tobacco. Information on smoking status was complete for 336 cases and 4,762 subcohort members.

Statistical analysis

Differences between cases with and without collected tumor material, between men and women and between cases with and without a mutation in the *VHL* gene were assessed by calculating student t-tests and chi-square tests. RRs were calculated for never, ex- and current smokers. All analyses were also carried out for men and women separately because of the difference in smoking habits between men and women. Most men were

current or ex-smokers while most women were never smokers in this cohort. Case groups were defined as follows: total RCC (all cases of RCC detected by linkage to cancer and pathology registry; N=337); clear-cell RCC (all cases of RCC classified as clear-cell after revision by one experienced pathologist (CAHK); N=187); *VHL* mutated clear-cell RCC (clear-cell RCC with a mutation in the *VHL* gene; N=114) and *VHL* wildtype clear-cell RCC (clear-cell RCC without a mutation in the *VHL* gene; N=73).

Furthermore, the association of smoking and specific type and location of mutations were investigated. Specifically, the total number of G:C→T:A[12] transversions, which has been linked to the carcinogenic tobacco smoke constituent benzo (a) pyrene diol epoxide (BPDE) and the number of G:C→A:T[11,12] transitions, which has been linked to N-nitrosamino compounds, another carcinogenic cigarette smoke constituent.

Confounders considered were age at baseline (years), sex, body mass index (kg/m^2), a history of hypertension (yes/no), a family history of RCC (yes/no), alcohol consumption (g/day), social economic status (SES) based on education, physical activity in leisure time (<30, 30-60, 60-90, >90 min/day), occupational physical activity (for men only) (<8, 8-12, >12 kj/min), vegetable (g/day) and fruit consumption (g/day) and energy intake (kcal/day). Those variables that were found to statistically significantly (p<0.05) contribute to the multivariable model for overall RCC (age at baseline and sex), using the Wald test, were included as covariates in multivariable analyses. Confounders entered in smoking status analyses were age, sex (if appropriate) and BMI. Cigar and pipe smoking (never, ex- and current smoker) were additionally included in the model to account for other sources of tobacco smoke. To assess dose response relations, the number of cigarettes smoked per day was categorized into <10, 10-20, 20-30 and 30 or more cigarettes per day (additionally adjusted for the number of smoking years), and the number of smoking years was categorized into <20, 20-40 and 40 or more smoking years (additionally adjusted for number of cigarettes smoked per day). Age at first exposure (<15, 15-17, 17-20 and 20 or older at first exposure) and years of cessation (<5, 5-20 or more than 20 years) were also investigated, with additional adjustment for the number of cigarettes smoked per day.

RRs and corresponding 95% confidence intervals (CI) were estimated using Cox proportional hazard models processed with STATA (STATA statistical software, Release 7, STATA Corporation, College Station, TX, USA, 2001), after testing the proportional hazards assumption using scaled Schoenfeld residuals[21]. Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort[22]. To obtain p values for dose-response trends, ordinal exposure variables were fitted as continuous terms. Two sided p values are reported throughout this paper.

Results

Table 5.1 shows baseline characteristics of potential confounders and smoking variables for subcohort members, all cases and cases with collected tumor material, separately for men and women. First, we checked whether the ability to collect tumor tissue introduced bias. There were no statistically significant differences in mean age, the proportion of men, or the distribution over the smoking categories never, ex- and current smokers (men and women combined) between cases with (N=235) or without (N=102) tumor tissue. The mean age was somewhat higher for cases than for subcohort members (table 5.1). A positive family history for RCC was seldomly reported, with only slight differences between subcohort members and cases. The distribution of cases in smoking categories differed between men and women; there were almost no male never smokers while female smokers were scarce.

Table 5.1 Description of possible confounding variables and smoking variables for men and women, Netherlands cohort study on diet and cancer, 1986-1997.

Variable	Men			Women		
	Subcohort	RCC cases	RCC, tumor tissue collected	Subcohort	RCC cases	RCC, tumor tissue collected
	N=2331	N=217	N=148	N=2431	N=119	N=87
Age - Mean (sd)	61.4 (4.2)	62.0 (3.8)	62.2 (3.8)	61.5 (4.3)	61.8 (4.0)	61.6 (4.0)
Family history of RCC - N (%)	14 (0.6)	3 (1.4)	2 (1.4)	33 (1.4)	1 (0.8)	1 (1.2)
History of hypertension - N (%)	534 (22.9)	56 (25.8)	37 (25.0)	700 (28.8)	41 (34.5)	31 (35.6)
	N=2247	N=210	N=141	N=2341	N=113	N=82
BMI - Mean (sd)	25.0 (2.6)	25.3 (2.7)	25.4 (2.6)	25.1 (3.6)	25.8 (3.4)	25.7 (3.2)
	N=2331	N=217	N=148	N=2431	N=119	N=87
Cigarette smoking– Never - N (%)	300 (12.9)	18 (8.3)	12 (8.1)	1,431(58.9)	66 (55.5)	49 (56.3)
Ex - N (%)	1,175(50.4)	108 (49.8)	79 (53.4)	491 (20.2)	21 (17.6)	15 (17.2)
Current - N (%)	856 (36.7)	91 (41.9)	57 (38.5)	509 (20.9)	32 (26.9)	23 (26.4)
Never smoker - N (%)	217 (9.3)	11 (5.1)	6 (4.1)	1431 (58.9)	64 (53.8)	48 (55.2)
Cigarette only smoker - N (%)	1413 (60.6)	136 (62.7)	86 (58.1)	995 (40.9)	53 (44.5)	38 (43.7)
Cigar and/or pipe smoker - N (%)	83 (3.6)	7 (3.2)	6 (4.1)	0	2 (1.7)	1 (1.2)
Cigarette and other type of tobacco smoker - N (%)	618 (26.5)	63 (29.0)	50 (33.8)	5 (0.2)	0	0

Table 5.2 shows multivariable adjusted analyses for smoking with different endpoints for the total group and stratified by sex. For women, cigar or pipe smoking were not included in the model since only 2 female cases smoked cigars (table 5.1). RRs are generally stronger for men, with statistically significantly increased risks for men only (table 5.2). However, no statistical significant interactions were observed for smoking

with sex. The RRs were higher for *VHL* wildtype clear-cell tumors than for mutated clear-cell tumors. This was observed both in men and women. The number of male never smokers, which was also the reference group, was very low for the molecular endpoints. Therefore these analyses were repeated using current smokers as a reference group to increase precision of estimates. Conclusions did not change (data not shown).

Table 5.2 Rate ratios for ex- and current smokers compared to never smokers for all tumors (total), clear-cell tumors, clear-cell tumors with a *VHL* gene mutation and *VHL* wildtype clear-cell cases, Netherlands cohort study on diet and cancer (1986-1997).

	Never smokers			Ex-smokers			Current smokers			
	Cases (N)	Person-years subcohort	RR[a]	Cases (N)	Person-years subcohort	RR (95% CI)	Cases (N)	Person-years subcohort	RR (95% CI)	*p* value for interaction[b]
Men & women[c]										
Total	81	17,906	1	125	16,759	1.17 (0.85-1.61)	117	13,377	1.60 (1.17-2.20)	0.43
Clear-cell	48	17,906	1	73	16,759	1.34 (0.91-1.99)	57	13,377	1.49 (0.98-2.26)	0.20
Clear-cell wildtype	18	17,906	1	27	16,759	1.58 (0.84-2.94)	26	13,377	2.06 (1.07-3.94)	0.55
Clear-cell mutated	30	17,906	1	46	16,759	1.20 (0.73-1.96)	31	13,377	1.17 (0.69-1.99)	0.24
Men[d]										
Total	17	3,086	1	105	11,643	1.52 (0.89-2.59)	88	8,176	2.07 (1.20-3.56)	
Clear-cell	6	3,086	1	60	11,643	2.43 (1.07-5.56)	39	8,176	2.54 (1.05-6.17)	
Clear-cell wildtype	2	3,086	1	21	11,643	2.68 (0.66-10.86)	15	8,176	2.95 (0.65-13.28)	
Clear-cell mutated	4	3,086	1	39	11,643	2.33 (0.84-6.44)	24	8,176	2.34 (0.79-6.94)	
Women[e]										
Total	64	14,820	1	20	5,116	0.95 (0.57-1.59)	29	5,201	1.37 (0.87-2.16)	
Clear-cell	42	14,820	1	13	5,116	0.94 (0.50-1.76)	18	5,201	1.29 (0.73-2.28)	
Clear-cell wildtype	16	14,820	1	6	5,116	1.12 (0.44-2.86)	11	5,201	2.04 (0.94-4.45)	
Clear-cell mutated	26	14,820	1	7	5,116	0.82 (0.35-1.92)	7	5,201	0.82 (0.35-1.93)	

[a] Reference group; [b] Test for interaction of sex and smoking; [c] Cigarette, cigar and pipe smoking, multivariable adjusted for age, sex and BMI; [d] Cigarette, cigar and pipe smoking, multivariable adjusted for age and BMI; [e] Cigarette smoking, multivariable adjusted for age and BMI.

Table 5.3 shows additional cigarette smoking features for men who smoke(d). The number of females who smoke(d) was too small for meaningful analyses. Risk of RCC increased with increasing smoking frequency (number of cigarettes/day) and to a lesser extent with smoking duration (the number of years smoked). Age at first exposure was not associated with RCC risk. The risk of RCC was mostly lower after cessation although no clear trend was present. There were no noteworthy differences between mutated and wildtype clear-cell RCC.
The distribution of smokers for the different type of mutations, the mutational spectra, specific mutations (i.e., G:C→T:A transversions and G:C→A:T transitions) and location of mutations is shown in table 5.4. There did not seem to be a difference between never, ex- and current smokers.

Discussion

We observed an increased risk of RCC with cigarette smoking for men, with a suggestion of an increasing risk with number of cigarettes smoked per day. This association was less clear for women. Risks were somewhat higher for tumors without a *VHL* gene mutation compared to tumors with one. This indicates that the number of mutations in the *VHL* gene was not increased by smoking, even though it has been clearly shown that cigarette smoking is associated with mutations in other genes such as *p53*.
Many studies have reported on smoking as a risk factor for RCC. Almost all concluded that smoking was associated with an increased risk and most observed increasing risks, not only with the number of cigarettes smoked per day but also with the number of years smoked and decreasing risks with number of years since cessation, as is also shown in the meta-analysis by Hunt *et al.*[9]. We observed a difference in risk between men and women, which was also observed in the meta-analysis[9]. This is probably the result of the difference in exposure between men and women, and maybe of different smoking habits. In the subcohort, female ex- and current smokers usually smoked filter-tipped cigarettes (60%), while male ex- and current smokers mainly smoked non-filtered cigarettes (65%).
The percentage of smokers in this cohort appears to be slightly lower compared to the percentage in the population. In the subcohort, 37% of men reported to be a current smoker while 21% of women reported to be current smokers. A report on percentages of male and female smokers in the age groups of 50-65 and 65+ in 1986 in the Netherlands reports 45% and 41% smokers for men and 29% and 12% smokers in women[23]. This difference may be the result of a selective response by smoking status to the baseline questionnaire or of underreporting of smoking habits because of social desirability. The response rate to the questionnaire at baseline equaled 35.5%[14], with a slight shift towards non-smoking compared to the population[24]. This selective response would not lead to altered RRs, while underreporting would result in an underestimation of the RRs.

Table 5.3 Additional cigarette smoking features for male current and ex-smokers for all tumors, for clear-cell tumors and for clear-cell tumors with or without a mutation, Netherlands cohort study on diet and cancer, 1986-1997.

		All tumors		Clear-cell		Clear-cell wildtype		Clear-cell mutated	
Cigarette smoking features	Person-years subcohort	Cases (N)	RR (95% CI)	Cases (N)	RR (95% CI)	Cases (N)	RR (95% CI)	Cases (N)	RR (95% CI)
Frequency (cigarettes/day)[a]									
<10	3,489	31	1 (reference)	14	1 (reference)	6	1 (reference)	8	1 (reference)
10-<20	6,643	58	0.93 (0.58-1.48)	28	1.01 (0.51-1.99)	10	0.84 (0.29-2.44)	18	1.13 (0.47-2.71)
20-<30	6,043	59	1.04 (0.65-1.67)	35	1.38 (0.70-2.70)	13	1.21 (0.44-3.37)	22	1.50 (0.62-3.62)
30+	1,957	29	1.52 (0.88-2.65)	16	1.77 (0.83-3.79)	6	1.61 (0.51-5.15)	10	1.89 (0.71-5.06)
p value for trend			0.16		0.08		0.32		0.14
Continuous per 10 cigarettes/day	18,132	177	1.14 (0.99-1.31)	93	1.19 (1.00-1.42)	35	1.26 (0.94-1.69)	58	1.16 (0.94-1.41)
Duration (years)[b]									
<20	2,625	21	1 (reference)	13	1 (reference)	4	1 (reference)	9	1 (reference)
20-<40	8,299	78	1.09 (0.66-1.80)	43	0.97 (0.51-1.84)	17	1.20 (0.40-3.60)	26	0.86 (0.39-1.89)
40+	7,208	78	1.25 (0.75-2.08)	37	1.03 (0.53-2.01)	14	1.20 (0.41-3.51)	23	0.96 (0.42-2.19)
p value for trend			0.33		0.86		0.80		0.99
Continuous per 10 years	18,132	177	1.10 (0.94-1.28)	93	1.07 (0.88-1.30)	35	1.10 (0.82-1.49)	58	1.05 (0.81-1.36)
Age at first exposure (years)[b]									
<15	4,073	41	1 (reference)	21	1 (reference)	8	1 (reference)	13	1 (reference)
15-<17	5,289	46	0.89 (0.57-1.39)	28	1.09 (0.61-1.96)	13	1.41 (0.58-3.48)	15	0.91 (0.43-1.92)
17-<20	5,371	51	0.96 (0.62-1.48)	24	0.89 (0.48-1.64)	7	0.70 (0.24-1.99)	17	0.99 (0.47-2.09)
20+	3,537	38	1.07 (0.66-1.72)	20	1.17 (0.61-2.24)	7	1.12 (0.39-3.20)	13	1.20 (0.54-2.67)
p value for trend			0.74		0.87		0.72		0.63
Years since cessation[b]									
Current smoker	7,212	77	1 (reference)	34	1 (reference)	14	1 (reference)	20	1 (reference)
<5	1,503	19	0.98 (0.56-1.71)	12	1.33 (0.66-2.65)	3	0.83 (0.24-2.85)	9	1.65 (0.71-3.81)
5-<20	6,059	46	0.58 (0.37-0.89)	26	0.68 (0.37-1.23)	9	0.59 (0.23-1.48)	17	0.75 (0.34-1.61)
20+	3,604	35	0.78 (0.50-1.21)	21	1.01 (0.56-1.83)	9	1.10 (0.47-2.61)	12	0.96 (0.44-2.12)
p value for trend			0.07		0.60		0.87		0.61

[a] Number of cigarettes smoked per day, years of cigarette smoking, pipe and cigar smoking, multivariable adjusted for age and BMI; [b] Number of smoking years or age at first exposure or years since cessation, number of cigarettes smoked per day, pipe and cigar smoking, multivariable adjusted for age and BMI.

Table 5.4 Distribution of smoking (never, ex- and current) for mutational characteristics of the *VHL* gene for all analyzed clear-cell tumors.

	Never smokers N (%)	Ex-smokers N (%)	Current smokers N (%)	*p* value
Type of mutation				
Point mutation	16 (32.0)	23 (46.0)	11 (22.0)	0.16[a]
Deletion or insertion	18 (26.9)	25 (37.3)	24 (35.8)	0.84[a]
Mutational spectra				
Silent	5 (38.5)	4 (30.8)	4 (30.8)	0.59[b]
Missense	7 (25.0)	16 (57.1)	5 (17.9)	0.09[a]
In-frame deletion/insertion	5 (33.3)	3 (20.0)	7 (46.7)	0.25[b]
Nonsense	1 (25.0)	2 (50.0)	1 (25.0)	1.00[b]
Frameshift deletion/insertion	14 (26.4)	21 (39.6)	18 (34.0)	1.00[a]
Truncating mutation	15 (26.3)	23 (40.4)	19 (33.3)	1.00[a]
Specific mutations				
G:C→T:A	3 (27.3)	5 (45.5)	3 (27.3)	0.93[b]
G:C→A:T	5 (31.3)	9 (56.3)	2 (12.5)	0.19[a]
Location				
Exon 1	18 (29.5)	24 (39.3)	19 (31.2)	0.83[a]
Exon 2	7 (25.9)	12 (44.4)	8 (29.6)	0.87[a]
Exon 3	6 (26.1)	9 (39.1)	8 (34.8)	1.00[a]
Intron	3 (33.3)	4 (44.4)	2 (22.2)	0.76[b]

[a] chi-square test of variable vs. remainder (other types/locations of mutations and *VHL* wildtype); [b] Fisher-Exact test because at least one expected value equals less than 2 or more than 20% of expected values equal less than 5.

Then the question remains whether we measured smoking at the most relevant period of life. Latency is unknown, but a latency of decades seems plausible. Most of the ever smokers in the subcohort had smoked for 20 years or more (86.0% of men and 75.5% of women), while 42.2% of men and 23.7% of women had smoked for 40 years or more. Also, 77.0% of men and 36.7% of women started smoking before the age of nineteen. These percentages are sufficiently high to assume that smoking could have caused cancer within the time frame measured by this study.

Because smoking is associated with risk of RCC and its constituents have been shown to cause mutations and the *VHL* tumor suppressor gene is considered to be one of the primary events in the carcinogenesis of RCC, we hypothesized smoking to be associated with *VHL* gene mutations. Contrary to what we expected, RRs were somewhat higher for *VHL* wildtype tumors than for *VHL* mutated tumors, regardless of sex. We also did not find an association of smoking and type of *VHL* gene mutations or with specific types of mutations implicated with exposure to cigarette smoke constituents. The observation that smoking, specifically exposure to BPDE, induces G:C→T:A transversions has predominantly been reported for the *p53* tumor suppressor

gene in lung cancer[25] and may not apply to all genes and cancer sites. For example, in a study on bladder cancer, not one *p53* G:C→T:A transversion was observed in smokers[26]. Exposure to N-nitrosamino compounds was associated with G:C→A:T transitions in the ras gene in tumors of rodents[12] and also with *VHL* gene mutations in rats[11]. We found no evidence to suggest this plays a role in humans.

To our knowledge, we are one of the first to investigate the association of risk factors with *VHL* gene mutations. A direct association between a risk factor and mutations may give additional information on the pathway(s) that lead to tumor growth. Previously, Brauch *et al.* observed a positive association of occupational exposure to trichloroethylene, an industrial solvent, to *VHL* gene mutations and a hot spot for mutations in a case-control study[6,7]. However, long-term exposure to an extremely high dose of the probable carcinogen trichloroethylene was investigated. Hemminki *et al.* investigated the association of vegetable and fruit consumption with mutations in a case-only study[8]. With regard to vegetable and fruit consumption, decreased risks were observed for vegetable consumption (for smokers) and citrus consumption (for smokers and smokers and non-smokers together) in 66 tests with the number of patients per group sometimes as low as 3[8]. In this report, the OR for risk of *VHL* gene mutations compared to wildtype *VHL* as a result of smoking equaled 0.95 (95% CI: 0.41-2.21) in a case-only analysis[8]. This supports our observation that smoking may not be associated with *VHL* gene mutations.

Smoking was associated with RCC risk for men but smoking was not associated with *VHL* gene mutations, irrespective of sex, implying that smoking may cause or promote RCC independent from *VHL* gene mutations.

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6

Hypertension, antihypertensives and mutations in the *von Hippel-Lindau* gene in renal cell carcinoma: results from the Netherlands cohort study

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Abstract

Objectives

Hypertension and/or antihypertensive medication are reported to be risk factors of renal cell carcinoma (RCC). We investigated whether these risk factors are associated with *von Hippel-Lindau* gene (*VHL*) mutations in RCC.

Methods

The Netherlands Cohort Study on Diet and Cancer (NLCS) started in 1986 (N=120,852 men and women) and uses the case-cohort methodology. After 11.3 years of follow-up, 337 RCC cases and 4,774 subcohort members were available for analysis. DNA was isolated from paraffin-embedded tumor tissue for *VHL* analysis.

Results

Cohort members who reported hypertension or use of anti-hypertensive medication had a slightly (non-significant) increased risk of RCC: rate ratios (RR) equaled 1.22 (95% CI: 0.94-1.58) and 1.14 (95% CI: 0.85-1.52), respectively. RRs were adjusted for sex, age, body mass index and cigarette smoking. Of the 235 patients for whom tumor tissue specimens were collected, 187 had a clear-cell RCC of whom 114 had a *VHL* mutation. History of hypertension was associated with a non-significantly increased risk of clear-cell RCC with *VHL* mutations (RR: 1.34; 95% CI:0.87-2.07), and was not associated with the risk of clear-cell RCC without *VHL* mutations (RR: 0.88; 95% CI:0.51-1.53). Use of diuretics was associated with clear-cell RCC without *VHL* mutations (RR: 2.11; 95% CI:1.16-3.83).

Conclusions

In this study non-significantly increased risks for history of hypertension and use of antihypertensive medication with RCC were observed. The association with hypertension was stronger in RCC patients with *VHL* mutations, while there was a positive association of diuretics use and risk of RCC without *VHL* mutations.

Introduction

Hypertension and use of diuretics or other antihypertensive medication have been found to be risk factors for renal cell carcinoma (RCC) in many epidemiological studies[1,2]. It is unclear, however, whether the increased risk is caused by hypertension itself, or by the use of antihypertensive medication. Some recent studies showed that diuretic medication was no longer a risk factor after controlling for the diagnosis of hypertension[3,4], suggesting that not medication but hypertension is a risk factor for RCC. Several prospective studies have studied the effect of hypertension on the risk of RCC[5-12], but few prospective cohort studies were also able to study the use of antihypertensive medication[8,11,12].

RCC is classified in different subtypes. The majority of RCC are of the clear cell type (~80%); other subtypes are papillary RCC (10%), chromophobe RCC (5%), collecting-duct carcinoma (1%) and unclassified RCC (3-5%)[13]. Von Hippel-Lindau (VHL) disease is a rare inherited disorder associated with (amongst others) an increased risk for clear-cell RCC[14]. After the identification of the *VHL* gene on chromosome 3p25 it became evident that this gene is also involved in the development of sporadic clear-cell RCC. It is estimated that approximately 75% of all sporadic clear-cell RCC harbor bi-allelic *VHL* defects[15]. Two studies suggested that risk factors, such as occupational exposure to trichloroethylene and fruit consumption, are associated with mutations in the *VHL* gene in RCC[16,17].

Despite the fact that many studies have observed an association between the diagnosis of hypertension and/or use of antihypertensive medication and RCC risk, there is still much uncertainty with respect to the biological mechanism. Gago-Dominguez[18] suggested the 'lipid peroxidation' hypothesis as the underlying mechanism, but this remains to be proven. The different subtypes of RCC may have different etiologies. The *VHL* gene is the main causative gene for sporadic clear-cell RCC[15]. Whether hypertension and/or use of antihypertensive medication are associated with clear-cell RCC, or more specifically with mutational status of the *VHL* gene, has not been investigated before. It is conceivable that these risk factors are associated with specific subtypes of RCC, or with mutational status of the *VHL* gene in clear-cell RCC.

We decided to study whether hypertension and use of antihypertensive medication were associated with risk of RCC, and more specifically with mutational status of the *VHL* gene in clear-cell RCC, within a large prospective cohort study.

Materials and methods

Subjects

The Netherlands Cohort Study on diet and cancer is a prospective cohort study, which started in September 1986. The study design has been reported in detail elsewhere[19]. Briefly, the cohort included 120,852 men and women, aged 55-69 years, at the

beginning of the study. The study was designed as a case-cohort study, using all cases and a random sample of 5,000 persons from the cohort (subcohort), who have been followed to estimate the accumulated person-years in the entire cohort[20]. Follow-up for incident cancer has been established by computerized record linkage with the Netherlands Cancer Registry and PALGA, a national database of pathology reports. The method of record linkage to obtain information on cancer incidence has been described previously[21]. The completeness of cancer follow-up was estimated to be more than 96%[22]. From 1986 to 1997 (11.3 years follow up) 355 kidney cancer cases [*International Classification of Diseases for Oncology*, version 3 (ICD-O-3): C64.9] were identified. Urothelial cell carcinomas were excluded and only histologically confirmed epithelial cancers were included (ICD-O: M8010-8119, 8140-8570), leaving 337 cases. All subcohort members who reported prevalent cancer (excluding skin cancer) at baseline were excluded from analyses (leaving 4,774 subcohort members).

VHL mutation analysis

Paraffin blocks of tumors were collected from 51 pathology laboratories, the procedures have been described in detail elsewhere[23]. We were able to collect material for 251 cases. One experienced pathologist (CAHK) revised all haematoxylin and eosin (HE)-stained slides. The RCCs were classified according to the World Health Organization (WHO) classification of Tumours of 2002[24]. The protocol for DNA isolation and mutation analyses have been described previously[23]. Briefly, paraffin was removed with xylene and tumor DNA was extracted by salt-precipitation. The entire gene was amplified using 6 primer sets as described before[23]. Samples were first subjected to polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP) analysis, which was followed by direct sequencing in case of aberrant or unclear results. Mutations were identified by visual inspection of sequences provided by the ABI basecaller (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands). After revision and *VHL* gene mutation analyses, data was available for 235 cases[23].

Questionnaire

At baseline, all cohort members completed a mailed, self-administered questionnaire on dietary habits and other risk factors for cancer[25]. Participants were asked to report whether a physician had ever diagnosed 'high blood pressure' and at what age the diagnosis was made (in five year age groups from 'younger than 30 years', in 30-34 years' to '65-69 years'). Duration since diagnosis was calculated by subtracting the midpoint age in the age group of diagnosis from the age at baseline and was categorized into three broad categories: 0-9 years, 10-19 years and ≥20 years.
Participants were also asked to report on use of any drugs that they used longer than six months, for what condition and in what calendar period. All drugs were classified into therapeutic groups using the Anatomical Therapeutic Chemical (ATC) classification of the WHO Collaborative Centre for Drug Statistical Methodology[26,27].

Data analysis

Differences between cases with and without collected tumor material were assessed by calculating Student t-tests and chi-square tests. RRs for RCC were calculated for history of hypertension and use of antihypertensive medication. Case groups were defined as follows: total RCC (all histologically confirmed cases of RCC detected by linkage to cancer and pathology registries; N=337); clear-cell RCC (classified as clear-cell RCC after pathological revision; N=187); mutated clear-cell RCC (clear-cell RCC with a mutation in the *VHL* gene; N=114) and wildtype clear-cell RCC (clear-cell RCC without a mutation in the *VHL* gene; N=73). Confounders considered were age at baseline (years), sex, current cigarette smoking (yes/no), cigarettes smoked (number/day), years of cigarette smoking (years), alcohol consumption (g/day), body mass index (BMI; kg/m^2), a history of diabetes mellitus (yes/no), a history of RCC in first-grade family (yes/no), non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), occupational physical activity for men only (<8, 8-12, >8 kj/min) and social economic status (SES) based on education. Those variables that were associated with diagnosis of hypertension (and/or use of antihypertensive medication), that were an independent risk factor of RCC and that changed the risk estimates for the association of hypertension (and/or the use of antihypertensive medication) and RCC more than 10% were included as confounders in multivariable analyses. Using these criteria, confounders entered in the analyses were age, sex, BMI, current cigarette smoking, number of cigarettes smoked per day, and number of years of cigarette smoking.
RRs and corresponding 95% confidence intervals (CI) for RCC were estimated using Cox proportional hazard models processed with the STATA statistical software package (STATA statistical software, Release 7, STATA Corporation, College Station, Texas, USA, 2001), after testing the proportional hazards assumption using scaled Schoenfeld residuals[28]. Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling person-time from the cohort[29]. To obtain *p* values for dose-response trends, ordinal exposure variables were fitted as continuous terms.

Results

Hypertension was reported somewhat more frequently among RCC cases than subcohort members (29.4% versus 26.3%; Table 6.1). RCC cases also reported a slightly higher use of antihypertensive medication than the subcohort members (23.2% versus 21.1%). RCC cases had a higher BMI, were more often current cigarette smokers and had been diagnosed less frequently with diabetes mellitus than subcohort members. When these variables were compared between patients with tumor material and patients without tumor material, no significant differences were observed. Only the percentage of antihypertensive medication use was significantly higher for cases with

tumor tissue collected compared to cases for whom no tissue could be collected (p=0.02).

Table 6.1 Description of exposure variables and potential confounders in subcohort members (N=4,774), RCC cases (N=337) and RCC cases with tissue blocks collected (N=235), The Netherlands Cohort Study on Diet and Cancer (NLCS), 1986-1997.

		Subcohort members (N=4,774) N (%)	RCC cases (N=337) N (%)	RCC cases with collected tumor material (N=235) N (%)
Hypertension and use of antihypertensive medication				
Diagnosis hypertension	No	3,517 (73.7)	238 (70.6)	166 (70.6)
	Yes	1,257 (26.3)	99 (29.4)	69 (29.4)
Duration since diagnosis[a,b]	0-9 yrs	664 (53.7)	56 (57.7)	35 (51.5)
	10-19 yrs	367 (29.7)	27 (27.8)	24 (35.3)
	20+ yrs	206 (16.7)	14 (14.4)	9 (13.2)
Ever use of hypertension medication	No	3,767 (78.9)	259 (76.9)	172 (73.2)
	Yes	1,007 (21.1)	78 (23.2)	63 (26.8)
Ever use of diuretics	No	4,238 (88.8)	298 (88.4)	206 (87.7)
	Yes	536 (11.2)	39 (11.6)	29 (12.3)
Ever use of beta-blockers	No	4,203 (88.0)	287 (85.2)	195 (83.0)
	Yes	571 (12.0)	50 (14.8)	40 (17.0)
Potentially confounding variables				
Age at baseline (years)[c]		61.4 (4.2)	61.9 (3.9)	62.0 (3.9)
BMI (kg/m^2)[c]		25.1 (3.1)	25.5 (3.0)	25.5 (2.9)
Current cigarette smoking[b]	No	3,397 (71.3)	213 (63.4)	155 (66.0)
	Yes	1,365 (28.7)	123 (36.6)	80 (34.0)
Number of cigarettes per day[c,d]		15.2 (10.2)	17.9 (12.3)	18.3 (12.4)
Years of smoking[c,d]		31.9 (12.3)	34.2 (12.2)	33.7 (12.1)
Alcohol consumption (g/day)[c]		10.4 (14.4)	11.5 (14.6)	10.6 (14.0)
Diagnosis of diabetes mellitus	No	4,588 (96.1)	326 (96.7)	226 (96.2)
	Yes	186 (3.9)	11 (3.3)	9 (3.8)
Family history of RCC	No	4,716 (99.0)	332 (98.8)	232 (98.7)
	Yes	47 (1.0)	4 (1.2)	3 (1.3)
Non-occupational physical activity[b]	<30 min/day	1,083 (23.2)	73 (22.1)	54 (23.4)
	30-<60 min/day	1,447 (30.9)	100 (30.2)	67 (29.0)
	60-<90 min/day	939 (20.1)	63 (19.0)	47 (20.4)
	≥90 min/day	1,210 (25.9)	95 (28.7)	63 (27.3)
Social economic status[b]	Primary school	1,476 (31.0)	94 (28.0)	64 (27.2)
	Lower vocational school	1,036 (21.8)	78 (23.2)	57 (24.3)
	Intermediate vocational school	1,584 (33.3)	109 (32.4)	74 (31.5)
	Higher vocational school or university	613 (12.9)	51 (15.2)	36 (15.3)

RCC= Renal cell carcinoma; BMI= Body Mass Index; [a] Only for persons who reported a diagnosis of hypertension; [b] Due to missing values totals do not add up to 4774, 337 and 235, respectively; [c] Mean (standard deviation); [d] Only for ever-smokers

Cohort members who reported a history of hypertension or use of antihypertensive medication at baseline, had a slightly increased risk of RCC (rate ratio (RR): 1.22; 95% confidence interval (95% CI): 0.94-1.58 and 1.14; (95% CI: 0.85-1.52), respectively

(Table 6.2). These RRs (as all following RRs) were adjusted for sex, age, BMI and cigarette smoking. There was no difference between men and women with respect to the association between history of hypertension and RCC risk (p for interaction = 0.99). A diagnosis of hypertension was associated with a RR of 1.21 (95% CI: 0.87-1.69) in men and a RR of 1.21 (95% CI: 0.80-1.84) in women. The RR for use of antihypertensive medication was also nearly the same in men and women (p for interaction = 0.92). With an increasing time interval between diagnosis of hypertension and baseline, hypertension was associated with slightly decreasing risks of RCC, with RRs of 1.27; 1.16 and 1.15, for time intervals of less than 10 years, 10 – 19 years, and 20 years or more, respectively.

Studying the interaction of the diagnosis of hypertension and antihypertensive medication use did not reveal divergent results. The RRs for use of diuretics or beta-blockers in cohort members, who had not reported a diagnosis of hypertension were relatively high, but the number of cases and person-years in the subcohort was small (Table 6.2).

Repeated analysis with exclusion of the first two years of follow-up did not alter the results considerably: RRs were slightly lower (data not shown).

Of the 235 patients for whom tissue specimens were available, 187 had a clear-cell RCC (80%). In 114 patients with clear-cell RCC a mutation in the *VHL* gene was detected (61%). The RR for diagnosis of hypertension was higher in cases with a *VHL* mutation than in cases with *VHL* wildtype: RRs equalled 1.34 (95% CI: 0.87-2.07) and 0.88 (95% CI: 0.51-1.53), respectively (Table 6.3). By contrast, the RR for use of antihypertensive medication was higher in cases with *VHL* wildtype (RR: 1.53; 95% CI: 0.89-2.61). The RR for use of diuretics was statistically significantly increased in cases with *VHL* wildtype (RR: 2.11; 95% CI: 1.16-3.83).

The RR was also increased in cohort members who used diuretics or beta-blockers and did not report a diagnosis of hypertension but numbers of cases and subcohort person-years were very small and the confidence intervals were wide.

Table 6.2 Age-adjusted and multivariable adjusted rate ratios (RR) for total RCC (N=337) according to hypertension and antihypertensive medication use; The Netherlands Cohort Study on Diet and Cancer (NLCS) 1986-1997.

Variable	Age- and sex-adjusted analyses			Multivariable adjusted analyses		
	N cases/ Person years subcohort	RR [a]	95% CI	N cases/ Person years subcohort	RR [b]	95% CI
Diagnosis of hypertension						
No	238 / 36,943	1	Ref	210 / 33,554	1	Ref
Yes	99 / 13,054	1.24	0.97-1.59	90 / 12,097	1.22	0.94-1.58
Time interval between diagnosis hypertension and baseline						
No diagnosis of hypertension	238 / 36,943	1	Ref	210 / 33,554	1	Ref
0-9 years	56 / 6,918	1.32	0.97-1.79	51 / 6,365	1.27	0.92-1.76
10-19 years	27 / 3,753	1.16	0.77-1.76	24 / 3,459	1.16	0.74-1.81
20+ years	14 / 2,181	1.06	0.61-1.86	14 / 2,075	1.15	0.66-2.03
p for trend			0.44			0.71
Antihypertensive medication use						
No	259 / 39,787	1	Ref	231 / 36,237	1	Ref
Yes	78 / 10,210	1.20	0.92-1.56	69 / 9,415	1.14	0.85-1.52
Diuretic use						
No	298 / 44,553	1	Ref	265 / 40,649	1	Ref
Yes	39 / 5,444	1.16	0.82-1.64	35 / 5,003	1.14	0.79-1.66
Beta-blocker use						
No	287 / 44,157	1	Ref	255 / 40,256	1	Ref
Yes	50 / 5,839	1.30	0.94-1.78	45 / 5,394	1.27	0.90-1.78
Diagnosis of hypertension and antihypertensive medication use						
No Hyp/No Med	219 / 34,310	1	Ref	194 / 31,148	1	Ref
No Hyp/Yes Med	19 / 2,633	1.06	0.65-1.73	16 / 2,408	0.95	0.56-1.64
Yes Hyp/No Med	40 / 5,478	1.19	0.83-1.68	37 / 5,090	1.16	0.80-1.68
Yes Hyp/Yes Med	59 / 7,576	1.29	0.95-1.75	53 / 7,008	1.25	0.91-1.74
Diagnosis of hypertension and diuretic use						
No Hyp/No Med	230 / 36,155	1	Ref	203 / 32,837	1	Ref
No Hyp/Yes Med	8 / 788	1.69	0.80-3.57	7 / 717	1.54	0.68-3.46
Yes Hyp/No Med	68 / 8,398	1.32	0.99-1.75	62 / 7,812	1.28	0.95-1.73
Yes Hyp/Yes Med	31 / 4,657	1.15	0.77-1.69	28 / 4,286	1.15	0.76-1.75
Diagnosis of hypertension and beta-blocker use						
No Hyp/No Med	222 / 35,348	1	Ref	196 / 32,098	1	Ref
No Hyp/Yes Med	16 / 1,595	1.41	0.82-2.41	14 / 1,456	1.36	0.76-2.44
Yes Hyp/No Med	65 / 8,811	1.23	0.92-1.64	59 / 8,159	1.21	0.89-1.64
Yes Hyp/Yes Med	34 / 4,243	1.33	0.91-1.95	31 / 3,939	1.31	0.87-1.96

RCC, renal cell carcinoma; RR, rate ratio; CI, confidence interval; Ref= reference; [a] Rate ratio adjusted for age (years) and sex; [b] Rate ratio adjusted for age (years), sex, body mass index (kg/m^2), current cigarette smoking at baseline (yes *versus* no), number of cigarettes smoked per day continuous) and years of cigarette smoking (continuous)

Table 6.3 Multivariable adjusted rate ratios (RR) for RCC with *VHL* mutation status according to hypertension and antihypertensive medication use. The Netherlands Cohort Study on Diet and Cancer (NLCS) 1986-1997.

Variable	Clear-cell RCC			Clear-cell RCC with a mutation in the *VHL*-gene			Clear-cell RCC, *VHL* wildtype		
	N cases/ Person years subcohort	Multivariable adjusted RR[a]	95% CI	N cases/ Person years subcohort	Multivariable adjusted RR[a]	95% CI	N cases/ Person years subcohort	Multivariable adjusted RR[a]	95% CI
Diagnosis of hypertension									
No	117 / 33,554	1	Ref	66 / 33,554	1	Ref	51 / 33,554	1	Ref
Yes	50 / 12,097	1.14	0.81-1.61	33 / 12,097	1.34	0.87-2.07	17 / 12,097	0.88	0.51-1.53
Time interval between diagnosis hypertension and baseline									
No hypertension	117 / 33,554	1	Ref	66 / 33,554	1	Ref	51 / 33,554	1	Ref
0-9 years	24 / 6,365	1.01	0.64-1.59	16 / 6,365	1.20	0.68-2.12	8 / 6,365	0.76	0.36-1.62
10-19 years	21 / 3,459	1.72	1.05-2.80	14 / 3,459	2.03	1.11-3.73	7 / 3,459	1.30	0.58-2.94
20+ years	5 / 2,075	0.69	0.28-1.72	3 / 2,075	0.74	0.23-2.36	2 / 2,075	0.64	0.16-2.64
p for trend			0.72			0.90			0.84
Antihypertensive medication use									
No	123 / 36,237	1	Ref	75 / 36,237	1	Ref	48 / 36,237	1	Ref
Yes	44 / 9,415	1.38	0.90-1.88	24 / 9,415	1.15	0.70-1.90	20 / 9,415	1.53	0.89-2.61
Diuretic use									
No	143 / 40,649	1	Ref	89 / 40,649	1	Ref	54 / 40,649	1	Ref
Yes	24 / 5,003	1.36	0.86-2.14	10 / 5,003	0.91	0.45-1.81	14 / 5,003	2.11	1.16-3.83
Beta-blocker use									
No	139 / 40,257	1	Ref	82 / 40,257	1	Ref	57 / 40,257	1	Ref
Yes	28 / 5,394	1.39	0.91-2.13	17 / 5,394	1.43	0.82-2.47	11 / 5,394	1.34	0.69-2.59
Diagnosis of hypertension and antihypertensive medication use									
No Hyp/No Med	103 / 31,147	1	Ref	59 / 31,147	1	Ref	44 / 31,147	1	Ref
No Hyp/Yes Med	14 / 2,407	1.53	0.85-2.78	7 / 2,407	1.32	0.59-2.98	7 / 2,407	1.82	0.78-4.22
Yes Hyp/No Med	20 / 5,090	1.12	0.69-1.84	16 / 5,090	1.57	0.90-2.75	4 / 5,090	0.53	0.19-1.48
Yes Hyp/Yes Med	30 / 7,008	1.25	0.81-1.92	17 / 7,008	1.24	0.69-2.21	13 / 7,008	1.26	0.68-2.34
Diagnosis of hypertension and diuretic use									
No Hyp/No Med	110 / 32,837	1	Ref	63 / 32,837	1	Ref	47 / 32,837	1	Ref
No Hyp/Yes Med	7 / 717	2.60	1.13-5.99	3 / 717	1.98	0.60-6.59	4 / 717	3.37	1.11-10.26
Yes Hyp/No Med	33 / 7,812	1.19	0.80-1.78	26 / 7,812	1.64	1.03-2.61	7 / 7,812	0.59	0.27-1.33
Yes Hyp/Yes Med	17 / 4,286	1.19	0.70-2.04	7 / 4,286	0.86	0.38-1.97	10 / 4,286	1.64	0.83-3.25
Diagnosis of hypertension and beta-blocker use									
No Hyp/No Med	60 / 32,098	1	Ref	60 / 32,098	1	Ref	45 / 32,098	1	Ref
No Hyp/Yes Med	6 / 1,456	2.23	1.18-4.22	6 / 1,456	1.91	0.80-4.56	6 / 1,456	2.67	1.09-6.57
Yes Hyp/No Med	22 / 8,159	1.23	0.83-1.83	22 / 8,159	1.40	0.85-2.30	12 / 8,159	1.00	0.53-1.90
Yes Hyp/Yes Med	11 / 3,939	1.17	0.67-2.02	11 / 3,939	1.41	0.71-2.77	5 / 3,939	0.85	0.34-2.15

RCC, renal cell carcinoma; RR, rate ratio; CI, confidence interval; Ref= reference; [a] All rate ratios are adjusted for age (years), sex, body mass index (kg/m^2), current cigarette smoking at baseline (yes *versus* no), number of cigarettes smoked per day (continuous) and years of cigarette smoking (continuous)

Discussion

In this study we observed that hypertension and use of antihypertensive medication were associated with a slightly increased, although statistically non-significant, risk of RCC. The association with hypertension was stronger in RCC patients with *VHL* mutations, while the association with use of antihypertensive medication was stronger in cases with *VHL* wildtype. Diuretics were associated with an increased risk of clear-cell RCC with *VHL* wildtype.

These results from the NLCS are most likely not affected by selection or information bias. Selection bias is unlikely given the high level of follow-up in terms of cases and subcohort person-years[22,30]. In theory, selection bias may have occurred in the collection of tissue samples[31]. For 235 of the 337 cases (70%), tumor material could be collected. There was no indication for bias in the selection of cases with tumor material according to the risk factors and potential confounders studied, except for the use of antihypertensive medication, which can be attributed most likely to chance. Information bias is unlikely in our study because the information with respect to the risk factors was collected before the diagnosis of RCC. Diagnosis of hypertension and use of antihypertensive medication were self-reported, however, and misclassification of exposure is a potential source of bias. In two studies conducted in the United States moderate agreement between self-report and actual measurement of blood pressure was observed; estimates of sensitivity for self-reported hypertension were between 62% and 82%[32] and 71% in the NHANES III study[33]. In an American validation study sensitivity of recall for use of antihypertensive medication among controls was 86% after two years and 79% after eight years[34]. In a small validation study (207 subjects) within our cohort study, use of medication for the cardiovascular system was recalled correctly by 66% of the users[26]. The subgroup of cases and subcohort members that reported use of antihypertensive medication, without reporting a diagnosis of hypertension, may indicate misclassification. Examining the indications for medication reported by the participants in the questionnaire at baseline revealed that only 11% of the persons in this group reported that the medication was used because of hypertension, suggesting that misclassification is limited. The misclassification of self-reported hypertension and use of antihypertensive medication is expected to be non-differential, attenuating the rate ratios towards one.

In a meta-analysis[2] based on 13 case-control studies a pooled adjusted odds ratio of 1.75 (95% CI: 1.61-1.90) was calculated for the association between hypertension and RCC. This pooled odds ratio did not include results from prospective cohort studies. Four prospective cohort studies measured blood pressure at baseline and followed the cohort members for the occurrence of RCC[5,6,9,10]. All studies found increased risks of RCC with increasing blood pressure. The outcomes of these studies are difficult to compare with our study, because of the different exposure measurement. Three prospective cohort studies used self-reported diagnosis of hypertension to define the exposure[8,11,12] and show therefore the highest resemblance with our study design. Two

of these studies reported increased RRs for diagnosis of hypertension and RCC risk[11,12]. These studies were relatively small, however, with 14 and 62 RCC cases, respectively. The Cancer Prevention Study II[8] included 1.2 million subjects and 335 RCC deaths after seven years of follow-up. Self-reported diagnosis of hypertension was associated with RCC deaths in females (RR: 2.2; 95% CI: 1.5-3.2), but not in males (RR: 1.1; 95% CI: 0.9-1.5)[8]. The RRs for hypertension observed in the current study are lower than the pooled odds ratio for case-control studies[2], but our estimates point in the same direction. Also, several other studies have published RRs comparable to ours, especially for males[4,8,35-37].

In another meta-analysis a pooled odds ratio was calculated for use of diuretics and risk of RCC[1]. Based on nine case-control studies an average odds ratio was calculated of 1.55 (95% CI: 1.42-1.71). Some of the included studies used self-report, while others used medical files or data from a pharmacy database[4,36,38,39]. Three prospective cohort studies also showed an increased risk for use of diuretics, with an exception for males in the Cancer Prevention Study II[7,8,11].

Hypertension may be a possible cause, but it may also be an early symptom of RCC[40]. The increased risk of hypertension may therefore reflect detection bias. Whether this bias is present may be evaluated by excluding cases from the analysis that were detected shortly after baseline measurement, or by investigating the relative risks according to time interval between diagnosis of hypertension and baseline. In our study, relative risks were slightly lower after exclusion of cases in the first two years of follow-up, and duration between diagnosis of hypertension and baseline was associated with slightly decreasing risks estimates. In a Danish record-linkage study[7] a U-shaped pattern was observed with highest risks for the shortest and the longest time interval. Other studies did not observe a modification of the risk estimates according to time interval[4,10,38,41,42].

Despite the large number of epidemiological studies, there is little convincing evidence with respect to the biological mechanism between hypertension or use of antihypertensive medication and the development of RCC. Gago-Dominguez *et al.*[18] suggested that lipid peroxidation, which is increased in obese and hypertensive individuals, might be responsible – at least in part – for the increased risk of RCC. By-products of lipid peroxidation have been shown to react with renal DNA to form adducts[18]. Diuretic therapy might be carcinogenic through conversion in the stomach to carcinogenic nitroso derivates or through a low-grade carcinogenic effect on the renal tubular cell, its principal target[1].

In our study, we also investigated whether hypertension and/or use of antihypertensive medication were associated with mutational status of the *VHL* gene. The *VHL* gene is a tumor suppressor gene. Loss of function is an early event in most cases of clear-cell RCC, by mutation or methylation of the promoter region. When hypertension and/or use of antihypertensive drugs are related to RCC risk, it is conceivable that these risk factors are associated with specific subtypes of RCC, e.g. clear-cell RCC with mutations in the *VHL* gene. In our analysis somewhat higher risks were found for

hypertension in relation to the risk of clear-cell RCC with a mutation in the *VHL* gene, and slightly decreased risks in cases with *VHL* wildtype. For use of antihypertensive medication and especially diuretic treatment, we observed the opposite; RRs were increased in cases with *VHL* wildtype and only slightly increased in cases with mutations in the *VHL* gene. It is possible that diagnosis of hypertension and use of diuretics and risk of clear-cell RCC work through different pathways (through a mutation in the *VHL* gene or not). However, false-positive findings because of multiple testing and small numbers cannot be excluded.
This is the first study to evaluate the association of hypertension and/or use of antihypertensive medication with mutations in the *VHL* gene. Without a specific *a priori* hypothesis it is difficult to exclude chance findings, especially with the small numbers in the sub-analyses. The findings with respect to the possible association with the *VHL* gene need to be confirmed in future studies.

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7

Carotenoid and vitamin intake, *von Hippel-Lindau* gene mutations and sporadic renal cell carcinoma: results from the Netherlands cohort study

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Abstract

Background
Carotenoids and vitamins may prevent cancer from occurring because of their antioxidant capacities. *Von Hippel-Lindau* (*VHL*) gene mutations are considered a primary event in the carcinogenesis of clear-cell renal cell carcinoma (RCC).

Objective
We investigated whether dietary carotenoid and vitamin intake and supplemental vitamin use were associated with RCC risk and with mutations in the *VHL* gene in clear-cell RCC.

Design
The Netherlands Cohort Study on diet and cancer (NLCS) includes 120,852 persons, who completed a self-administered food-frequency questionnaire in 1986. After 11.3 years of follow-up, 314 cases and a random sample of 4,438 persons (subcohort) with complete dietary data were used in a case-cohort approach. *VHL* gene mutational analysis was complete for 225 cases. Rate ratios (RRs) and corresponding 95% confidence intervals were estimated using Cox proportional hazard models, while adjusting for age, sex, smoking, body mass index and a history of hypertension.

Results
We observed no association for dietary carotenoid and vitamin intake and RCC risk. We observed a somewhat increased risk with supplemental vitamin E, AD, and multivitamin use. Results were suggestive of higher RRs for alpha-carotene, beta-cryptoxanthin, folate, and supplemental vitamin C and multivitamin intake for wildtype *VHL* tumors.

Conclusions
There was no association of carotenoid, vitamin or supplemental vitamin intake and RCC risk. Some results were suggestive for a differential effect between *VHL* wildtype and *VHL* mutated tumors. These associations should be investigated by others to confirm the current observations.

Introduction

Renal Cell Carcinoma (RCC) is the ninth most common tumor in the European Union[1]. Both the incidence and mortality rates are about twice as high for males than females[1]. The 5-year survival is approximately 55%[2].

Mutations in the *von Hippel-Lindau* (*VHL*) gene are believed to be an early event in renal carcinogenesis. *VHL* mutations are mainly observed in tumors of the most common histological subtype, i.e. clear-cell RCC[3]. Mutations are observed in the entire gene and usually lead to a truncated inactive protein[3]. The *VHL* gene is a tumor suppressor gene involved in cell cycle regulation, regulation of hypoxia inducible genes and proper fibronectin assembly in extracellular matrix[4,5]. It is estimated that 56% to 69%[4] of clear-cell renal tumors harbor a mutation in the *VHL* gene.

Occupational exposure to trichloroethylene[6,7] and consumption of citrus fruit and vegetables (confined to smokers)[8] have been linked to *VHL* gene mutations in renal cell carcinoma (RCC) in previous studies[6-8]. There is some evidence that the intake of vegetables, selenium and particularly citrus fruit protects the renal *VHL* gene from mutational insults, although chance results could not be ruled out[8]. To our knowledge, there are no other reports on an association of fruits and vegetables, or the more specific carotenoids and vitamin intake, and *VHL* gene mutations published thus far.

Generally, intake of carotenoids and vitamins are considered to be protective of cancer, mainly due to their ascribed anti-oxidant capacities[9]. In a study by Nyberg *et al.* diet and smoking habits were linked to somatic mutations in vivo[10]. The mutant frequency was significantly decreased in relation to vitamin C intake, while a u-shaped association with higher mutation frequencies at lower and higher intakes was observed with dietary carotenoid intake[10]. Dietary antioxidants take part in cellular reduction-oxidation (redox) reactions in which they can act as either antioxidants (electron donors) or prooxidants (electron acceptors), depending on the physiological environment and general oxidative state[11]. Thus, the possibility exists that, in an environment resulting in prooxidant activity by dietary antioxidants, antioxidant supplementation may actually cause harm in terms of increased risk of new disease[11].

A limited number of epidemiological studies investigated the association of carotenoids and vitamins and risk of RCC[12-21]. Null associations have been observed for vitamin A intake[13,15,16,21], inverse associations[15,20,21] but also null associations for carotenoids[13,16], inverse associations for vitamin C intake[15,20] and null[16] and inverse[13] associations for vitamin E intake. A reduced risk was observed for vitamin E[14] and multivitamin supplementation[13], while no protective effect was shown for beta-carotene supplementation[12].

We investigated whether dietary carotenoid (alpha-carotene, beta-carotene, lutein+zeaxanthine, beta-cryptoxanthine, and lycopene), and vitamin (vitamins A (retinol), C, E and folate) intake and supplemental vitamin C, E, AD, and multivitamin use were associated with sporadic RCC and, more specifically, with *VHL* gene mutations in clear-cell RCC in a prospective cohort study. Stratified analyses by

smoking were also carried out since a differential effect by smoking status has been suggested for the association of citrus fruits and RCC and *VHL* gene mutations[8], and for the association of some carotenoids and vitamins and RCC[16].

Subjects and methods

Netherlands Cohort Study

The Netherlands Cohort Study on diet and cancer (NLCS) started in September 1986. The study design has been reported in detail elsewhere[22]. Briefly, the cohort included 120,852 men and women aged 55-69 years in 1986. The study was designed as a case-cohort study, using all cases and a random sample of 5,000 persons from the cohort (subcohort), who have been followed for vital status information to estimate the accumulated person-years in the entire cohort[23].

Follow-up for incident cancers and vital status

The entire cohort was followed for incident cancer by computerized record linkage with the Netherlands Cancer Registry and PALGA, a national database of pathology reports. All participants who reported prevalent cancer (excluding skin cancer) at baseline were excluded from analyses (leaving 4,774 subcohort members). The method of record linkage to obtain information on cancer incidence has been described previously[24]. The completeness of cancer follow-up was estimated to be more than 96%[25]. From 1986 to 1997 (11.3 years follow up) 355 kidney cancer cases (ICD-O-3: C64.9) were identified. Urothelial cell carcinomas were excluded and only histologically confirmed epithelial cancers were included (ICD-O: M8010-8119, 8140-8570), leaving 337 cases.
The subcohort has been followed up for vital status information biennially by mail. The vital status of subcohort members who did not respond, was completed by contacting municipal population registries. Only two male subcohort members were lost to follow-up after 11.3 years of follow-up.

Questionnaire

At baseline, all cohort members completed a mailed, self-administered questionnaire on dietary habits, lifestyle, smoking, personal and family history of cancer and demographic data[26]. The questionnaire concentrated on the habitual consumption of food and beverages during the year preceding the start of the study. The dietary section of the questionnaire was a 150-item semi-quantitative food-frequency questionnaire, which was validated against 3-day diaries completed at three time points during a calendar year[26]. The correlation, adjusted for error index and day to day variation, between the record and the questionnaire was estimated at 0.76 for vitamin A intake and at 0.58 for vitamin C intake[26].

Mean daily nutrient intakes were calculated using the computerized Dutch food composition table[27]. For calculation of the intake of specific carotenoids, an additional food composition table has been constructed[28], providing information on alpha-carotene, beta-carotene, lutein+zeaxanthine, beta-cryptoxanthine and lycopene. Briefly, foods that are the main sources of carotenoids (e.g., vegetables) and some other foods were sampled and analyzed for alpha-carotene, beta-carotene, lutein, zeaxanthin, and lycopene. Values for all other foods were mostly derived from recent literature with the same methods of analysis used. In the carotenoid food composition table, lutein and zeaxanthin had to be taken together because most of the literature sources did not provide separate values for each of these carotenoids[28]. Folate data were derived from a validated liquid chromatography trienzyme method[29], used to analyze the 125 most important Dutch foods contributing to folate intake[30].
Information on supplement use was collected with an open-ended question with space for four different supplements at most. Participants were asked whether they used vitamin tablets, drops, or other preparations during the 5 years before baseline[31]. The relative validity of this open-ended question was studied in comparison to reference information from three personal interviews carried out within a period of 10 months[32]; recall for overall vitamin supplement use was 72.7%[32]. In that study vitamin supplement use included vitamin A, C, AD, B1, B2, B6, B12, B complex, E and multivitamin intake. In the current study, we investigated supplemental vitamin C, E, A and/ or D (AD) and multivitamin intake.
According to criteria published before[26], subjects with incomplete or inconsistent dietary data were excluded; 314 RCC cases and 4,438 subcohort members remained for analyses.

VHL gene mutation analysis

Paraffin material of cancer cases was collected after approval by the Medical Ethical Committees of Maastricht University, PALGA and the Netherlands cancer registry. We were able to collect paraffin blocks of tumors for 251 cases from 51 pathology laboratories, which we described in detail elsewhere[33].
One experienced pathologist (CAHK) revised all HE-stained slides. The RCC were classified according to the World Health Organization (WHO) classification of Tumours of 2002[34]. DNA isolation and mutation analyses have been described previously. Briefly, paraffin was removed with xylene and DNA was extracted by salt-precipitation. The entire gene was amplified using 6 primer sets. Samples were first subjected to PCR-SSCP analysis, which was followed by direct sequencing in case of aberrant or equivocal results. Mutations were identified by visual inspection of sequences provided by the ABI basecaller. After revision and *VHL* gene mutation analyses, data was available for 235 cases[33]. Information for 10 cases was discarded, since the dietary data for these subjects was incomplete or inconsistent.

Statistical analysis

Based on the literature and previous analyses, considered confounders were age at baseline (years), sex, body mass index (kg/m^2), a history of hypertension (yes/no), a family history of RCC (yes/no), alcohol consumption (g/day), social economic status (SES) based on education, non-occupational physical activity (<30, 30-60, 60-90, >90 min/day), occupational physical activity (for men only) (<8, 8-12, >12 kj/min), and energy intake (kcal/day). Those variables that were associated with RCC and were correlated with one of the carotenoids or vitamins were included as covariates in multivariable analyses. Confounders entered in these multivariable analyses were age, sex, smoking, BMI and a history of hypertension.

Differences in age, sex, smoking status, BMI and a history of hypertension between cases with (N=225) and without (N=89) collected tumor material were assessed by calculating student t-tests and chi-square tests.

RRs were calculated for the dietary intake of carotenoids alpha-carotene, beta-carotene, lutein+zeaxanthine, beta-cryptoxanthine and lycopene, and of the vitamins A (retinol), C, E and folate. Exposure variables were categorized into quintiles based on the distribution in the subcohort, for men and women separately. For vitamin C, however, quintiles 2 and 3 and quintiles 4 and 5 were combined, because the validation study demonstrated that these quintiles could not be distinguished[26]. Furthermore, we investigated use of supplemental vitamin C, E, AD and multivitamins (yes or no).

Analyses were carried out for the following case groups: total RCC (all cases of RCC detected by linkage to cancer and pathology registry; N=314); clear-cell RCC (all cases of RCC classified as clear-cell after revision by one experienced pathologist (CAHK); N=179); *VHL* mutated clear-cell RCC (clear-cell RCC with a mutation in the *VHL* gene; N=110) and *VHL* wildtype clear-cell RCC (clear-cell RCC without a mutation in the *VHL* gene; N=69).

RRs and corresponding 95% confidence intervals (CI) were estimated using Cox proportional hazard models processed with STATA (STATA statistical software, Release 7, STATA Corporation, College Station, TX, USA, 2001), after testing the proportional hazards assumption using scaled Schoenfeld residuals[35]. Standard errors were estimated using the robust Huber-White sandwich estimator to account for additional variance introduced by sampling from the cohort[36]. To obtain *p* values for dose-response trends, ordinal exposure variables were fitted as continuous terms. Two sided *p* values are reported throughout this paper.

Results

Baseline characteristics for exposure variables and potential confounders for subcohort members, all cases, cases with collected tumor material, clear-cell cases and clear-cell cases with or without a *VHL* gene mutation are shown in table 7.1. First, we checked for differences in characteristics of RCC cases for whom we could (N=225) or could

not (N=89) collect tumor tissue. There were no differences in mean age (p=0.62), the percentage of men (p=0.16), the percentage of smokers (never, ex and current) (p=0.33), mean BMI (p=0.85), or the percentage of cases that reported a history of hypertension (p=0.71).

We also tested differences in mean carotenoid and vitamin intake and in the percentage of supplement users between clear-cell RCC with and without a *VHL* gene mutation. There were no remarkable differences in mean carotenoid and vitamin intake between clear-cell RCC with and clear-cell RCC without a *VHL* gene mutation (results not shown). The percentage of supplemental vitamin C and multivitamin users was higher for clear-cell RCC with wildtype *VHL* compared to clear-cell RCC with a *VHL* gene mutation. (10.1% versus 4.6% (p=0.15) and 10.1% versus 3.6% (p=0.08), respectively), although these differences were statistically not significant.

RRs for the association of carotenoids, vitamins, and supplement use and total RCC, clear-cell RCC and clear-cell RCC with or without *VHL* gene mutations are shown in table 2. A statistically significant inverse association with RCC was observed in quintile 2 of lutein+zeaxanthin intake. Furthermore, a statistically significant increased risk of RCC was observed in quintile 4 of beta-cryptoxanthin intake. Eyeballing these results, we observed a possible u-shaped association of carotenoids and RCC risk, with the exception of beta-cryptoxanthin and lycopene. Higher intakes of beta-cryptoxanthin and lycopene were associated with an increased RCC risk. The possible u-shaped association was no longer present when investigating the association of carotenoids and clear-cell RCC risk. The observed RRs for wildtype *VHL* tumors were higher than those observed for *VHL* mutated tumors in case of dietary alpha-carotene and beta-cryptoxanthin intake (table 7.2). Vitamin intake did not seem to be associated with RCC risk, but there was a possible differential effect for folate intake between tumors with and without a *VHL* gene mutation. RRs were mostly greater than 1 in case of wildtype *VHL* tumors, while these were mostly lower than 1 for *VHL* mutated tumors. Supplemental vitamin E, AD and multivitamin use were associated with increased RCC risk, while there was no association of supplemental vitamin C use and RCC risk. Stratified analyses based on *VHL* mutational status revealed higher RRs for wildtype tumors in case of supplemental vitamin C and multivitamin use and lower RRs for wildtype tumors in case of supplemental vitamin E and AD use. Supplemental multivitamin use was associated with a statistically significant increased risk or clear-cell tumors without a *VHL* gene mutation (RR: 2.51; 95% CI: 1.10-5.75).

Table 7.1 Descriptives for carotenoids, vitamins, supplement use and confounding factors for subcohort members and case groups. Netherlands cohort study on diet and cancer, 1986-1997.

	Subcohort	RCC - total	Tumor material collected	Clear-cell RCC	Clear-cell RCC *VHL* mutated	Clear-cell RCC wildtype *VHL*
	N=4438	N=314	N=225	N=179	N=110	N=69
Carotenoids						
Alpha-carotene (mg/day) - Mean (sd)	0.696 (0.569)	0.685 (0.495)	0.666 (0.444)	0.676 (0.462)	0.674 (0.478)	0.680 (0.439)
Beta-carotene (mg/day) - Mean (sd)	2.96 (1.57)	2.97 (1.44)	2.91 (1.24)	2.94 (1.31)	2.94 (1.36)	2.93 (1.25)
Lutein+Zeaxanthin (mg/day) - Mean (sd)	2.50 (1.13)	2.56 (1.11)	2.50 (1.03)	2.52 (1.06)	2.57 (1.11)	2.44 (0.991)
Beta-cryptoxanthin (mg/day) - Mean (sd)	0.179 (0.179)	0.186 (0.179)	0.185 (0.172)	0.188 (0.168)	0.186 (0.168)	0.191 (0.169)
Lycopene (mg/day) - Mean (sd)	1.20 (1.77)	1.18 (1.88)	1.26 (2.15)	1.25 (2.21)	1.13 (1.28)	1.45 (3.17)
Dietary vitamins						
Vitamin A (retinol) (mg/day) - Mean (sd)	0.970 (0.418)	0.977 (0.429)	0.967 (0.428)	0.977 (0.453)	0.981 (0.455)	0.971 (0.454)
Vitamin C (mg/day) - Mean (sd)	103 (43.8)	103 (44.8)	102 (43.8)	102 (43.6)	101 (41.9)	103 (46.6)
Vitamin E (mg/day) - Mean (sd)	13.4 (6.19)	13.9 (6.81)	13.6 (6.14)	13.6 (6.29)	13.4(6.44)	13.8 (6.09)
Folate (mg/day) - Mean (sd)	0.211 (0.0722)	0.214 (0.0771)	0.211 (0.0755)	0.211 (0.0780)	0.208 (0.0712)	0.215 (0.0881)
Vitamin supplements						
Supplemental vitamin C user - N (%)	289 (6.5)	19 (6.1)	15 (6.7)	12 (6.7)	5 (4.6)	7 (10.1)
Supplemental vitamin E user - N (%)	87 (2.0)	9 (2.9)	5 (2.2)	4 (2.2)	3 (2.7)	1 (1.5)
Supplemental vitamin AD user - N (%)	130 (2.9)	11 (3.5)	6 (2.7)	5 (2.8)	3 (2.7)	2 (2.9)
Supplemental multivitamin use - N (%)	207 (4.7)	21 (6.7)	16 (7.1)	11 (6.2)	4 (3.6)	7 (10.1)
Confounding factors						
Age - Mean (sd)	61.4 (4.23)	61.9 (3.88)	62.0 (3.87)	61.7 (3.82)	61.8 (3.76)	61.5 (3.92)
Sex = Male - N (%)	2191 (49.4)	207 (65.9)	143 (63.6)	108 (60.3)	69 (62.7)	39 (56.5)
Cigarette smoker						
Never - N (%)	1590 (35.8)	78 (24.8)	58 (25.8)	47 (26.3)	31 (28.2)	16 (23.2)
Ex - N (%)	1593 (35.9)	122 (38.9)	91 (40.4)	74 (41.3)	47 (42.7)	27 (39.1)
Current - N (%)	1255 (28.3)	114 (36.3)	76 (33.8)	58 (32.4)	32 (29.1)	26 (37.7)
Number in analyses[a]	N=4272	N=293	N=209	N=168	N=102	N=66
Cigarettes smoked per day - Mean (sd)	9.51 (10.9)	13.1 (13.1)	13.3 (13.4)	13.7 (13.9)	13.6 (13.4)	13.8 (14.8)
Number in analyses[a]	N=4380	N=310	N=223	N=177	N=109	N=68
Years of cigarette smoking - Mean (sd)	20.2 (18.2)	25.5 (18.2)	24.8 (18.1)	24.2 (18.0)	23.6 (18.0)	25.2 (17.9)
Kidney cancer in first-degee relatives - N (%)	43 (1.0)	3 (1.0)	3 (1.3)	2 (1.1)	2 (1.8)	0
Number in analyses[a]	N=4292	N=304	N=215	N=172	N=104	N=68
BMI (kg/m^2) – Mean (sd)	25.0 (3.12)	25.5 (2.95)	25.5 (2.89)	25.8 (3.00)	25.8 (3.05)	25.8 (2.93)
History of hypertension - N (%)	1156 (26.1)	94 (29.9)	66 (29.3)	52 (29.1)	35 (31.8)	17 (24.6)

[a] The number of cases was lower in analyses for number of cigarettes smoked per day, number of years of cigarette smoking, and BMI due to missing values.

Table 7.2 Relative risks for the association of dietary carotenoid and vitamin intake, and supplemental vitamin use and Renal Cell Carcinoma (RCC), clear-cell RCC and clear-cell RCC with or without a *von Hippel-Lindau* gene mutation, Netherlands cohort study on diet and cancer 1986-1997.

Carotenoid/Vitamin	Median intake in subcohort Men / Women	RCC RR (95% CI)	Clear-cell RCC RR (95% CI)	Clear-cell RCC *VHL* gene mutated RR (95% CI)	Clear-cell RCC Wildtype RR (95% CI)
Number of cases		284	162	97	65
Number person-years in subcohort		42,972	42,972	42,972	42,972
Alpha-carotene					
Q1	0.19 / 0.18	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	0.38 / 0.37	0.85 (0.59-1.24)	0.81 (0.49-1.34)	0.67 (0.35-1.28)	1.07 (0.48-2.38)
Q3	0.57/ 0.56	0.82 (0.56-1.20)	0.97 (0.60-1.57)	0.93 (0.51-1.68)	1.04 (0.47-2.32)
Q4	0.82 / 0.82	0.76 (0.52-1.12)	0.76 (0.45-1.26)	0.61 (0.31-1.17)	1.05 (0.47-2.34)
Q5	1.31 / 1.32	0.90 (0.62-1.31)	0.96 (0.59-1.57)	0.84 (0.45-1.55)	1.20 (0.54-2.65)
p trend		0.46	0.80	0.53	0.71
Continuous per 0.1 mg/day		0.99 (0.97-1.01)	0.98 (0.96-1.01)	0.98 (0.95-1.02)	0.99 (0.95-1.03)
Beta-carotene					
Q1	1.48 / 1.39	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	2.14 / 2.03	0.76 (0.51-1.12)	0.92 (0.54-1.55)	0.89 (0.45-1.74)	0.96 (0.43-2.17)
Q3	2.67 / 2.61	0.84 (0.57-1.23)	1.22 (0.74-2.01)	1.26 (0.67-2.36)	1.17 (0.53-2.56)
Q4	3.37 / 3.32	0.84 (0.57-1.23)	1.01 (0.61-1.69)	1.09 (0.58-2.07)	0.89 (0.39-2.04)
Q5	4.75 / 4.72	0.97 (0.67-1.40)	1.09 (0.66-1.82)	0.94 (0.48-1.83)	1.34 (0.62-2.89)
p trend		0.95	0.64	0.92	0.54
Continuous per 1 mg/day		0.98 (0.91-1.06)	0.96 (0.87-1.05)	0.95 (0.84-1.07)	0.98 (0.85-1.12)
Lutein+Zeaxanthin					
Q1	1.42 / 1.30	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	1.89 / 1.81	0.66 (0.45-0.99)	0.68 (0.41-1.13)	0.57 (0.28-1.14)	0.84 (0.40-1.79)
Q3	2.37 / 2.29	0.79 (0.54-1.16)	0.81 (0.49-1.31)	0.78 (0.41-1.46)	0.85 (0.40-1.79)
Q4	2.86 / 2.78	0.92 (0.64-1.33)	0.99 (0.62-1.58)	1.12 (0.63-2.02)	0.79 (0.37-1.68)
Q5	3.89 / 3.77	0.90 (0.62-1.29)	0.76 (0.46-1.25)	0.76 (0.40-1.45)	0.76 (0.35-1.63)
p trend		0.88	0.74	0.89	0.48
Continuous per 1 mg/day		1.01 (0.91-1.12)	0.96 (0.84-1.10)	0.98 (0.81-1.17)	0.94 (0.76-1.15)
Beta-cryptoxanthin					
Q1	0.01 / 0.03	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	0.04 / 0.09	1.08 (0.72-1.62)	1.24 (0.72-2.13)	1.00 (0.52-1.93)	1.88 (0.73-4.82)
Q3	0.10 / 0.17	0.99 (0.65-1.50)	1.11 (0.64-1.93)	0.63 (0.30-1.32)	2.40 (0.97-5.95)
Q4	0.20 / 0.27	1.56 (1.06-2.28)	1.58 (0.95-2.65)	1.18 (0.63-2.22)	2.64 (1.08-6.46)
Q5	0.36 / 0.50	1.17 (0.78-1.74)	1.40 (0.83-2.36)	1.30 (0.70-2.42)	1.61 (0.62-4.16)
p trend		0.11	0.11	0.31	0.18
Continuous per 0.05 mg/day		1.03 (1.00-1.06)	1.03 (0.99-1.07)	1.03 (0.98-1.08)	1.03 (0.97-1.09)
Lycopene					
Q1	0.14 / 0.17	1 (reference)	1 (reference)	1 (reference)	1(reference)
Q2	0.42 / 0.56	0.90 (0.59-1.35)	0.68 (0.38-1.21)	0.81 (0.41-1.63)	0.45 (0.16-1.29)
Q3	0.74 / 0.90	1.12 (0.76-1.67)	1.21 (0.73-2.00)	1.10 (0.58-2.09)	1.40 (0.64-3.06)
Q4	1.11 / 1.30	1.35 (0.93-1.98)	1.47 (0.90-2.38)	1.26 (0.68-2.36)	1.81 (0.86-3.82)
Q5	1.98 / 2.33	1.17 (0.79-1.72)	1.23 (0.74-2.04)	1.13 (0.59-2.16)	1.39 (0.64-3.05)
p trend		0.10	0.05	0.36	0.04
Continuous per 0.5 mg/day		1.01 (0.88-1.17)	1.05 (0.88-1.25)	0.96 (0.79-1.18)	1.13 (0.89-1.44)

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Carotenoid/Vitamin	Median intake in subcohort Men / Women	RCC RR (95% CI)	Clear-cell RCC RR (95% CI)	Clear-cell RCC *VHL* gene mutated RR (95% CI)	Clear-cell RCC Wildtype RR (95% CI)
Number of cases		284	162	97	65
Number person-years in subcohort		42,972	42,972	42,972	42,972
Vitamin A (retinol)					
Q1	0.61 / 0.52	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	0.81 / 0.70	1.01 (0.69-1.48)	0.96 (0.58-1.60)	1.11 (0.59-2.10)	0.76 (0.34-1.72)
Q3	0.95 / 0.84	1.00 (0.68-1.47)	1.12 (0.69-1.84)	1.14 (0.60-2.14)	1.11 (0.52-2.36)
Q4	1.14 / 1.01	0.73 (0.48-1.11)	0.75 (0.44-1.29)	0.89 (0.45-1.75)	0.57 (0.23-1.37)
Q5	1.51 / 1.37	1.13 (0.78-1.64)	1.05 (0.64-1.72)	0.94 (0.49-1.81)	1.20 (0.58-2.48)
p trend		0.96	0.84	0.63	0.81
Continuous per 0.1 mg/day		0.99 (0.96-1.03)	1.00 (0.95-1.04)	1.00 (0.94-1.05)	1.00 (0.94-1.06)
Vitamin C					
Q1	52.23 / 58.93	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2 + Q3	81.78 / 92.73	1.10 (0.78-1.55)	1.04 (0.67-1.60)	0.90 (0.52-1.56)	1.28 (0.65-2.55)
Q4 + Q5	129.76 / 140.84	1.01 (0.72-1.43)	0.88 (0.57-1.38)	0.87 (0.50-1.50)	0.91 (0.44-1.87)
p trend		0.99	0.48	0.63	0.58
Continuous per 10 mg/day		1.01 (0.98-1.04)	0.99 (0.96-1.03)	0.99 (0.94-1.04)	1.00 (0.94-1.06)
Vitamin E					
Q1	7.18 / 6.13	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	10.56 / 8.54	0.77 (0.51-1.16)	0.66 (0.38-1.15)	0.91 (0.46-1.78)	0.35 (0.12-0.97)
Q3	13.54 / 11.05	1.09 (0.75-1.58)	1.16 (0.72-1.88)	1.53 (0.84-2.80)	0.69 (0.30-1.57)
Q4	17.20 / 14.40	1.04 (0.72-1.52)	1.13 (0.70-1.81)	0.74 (0.37-1.49)	1.63 (0.85-3.13)
Q5	23.76 / 19.55	1.00 (0.68-1.47)	0.90 (0.54-1.50)	1.00 (0.52-1.93)	0.76 (0.34-1.70)
p trend		0.53	0.63	0.81	0.30
Continuous per 5 mg/day		1.05 (0.95-1.15)	1.03 (0.90-1.16)	1.00 (0.84-1.19)	1.07 (0.89-1.27)
Folate					
Q1	0.15 / 0.13	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Q2	0.18 / 0.16	0.83 (0.56-1.24)	0.74 (0.44-1.24)	0.62 (0.33-1.19)	0.99 (0.42-2.34)
Q3	0.21 / 0.19	1.04 (0.72-1.52)	1.08 (0.74-1.77)	1.02 (0.58-1.80)	1.20 (0.54-2.66)
Q4	0.25 / 0.22	0.86 (0.58-1.27)	0.81 (0.49-1.35)	0.52 (0.26-1.05)	1.44 (0.66-3.14)
Q5	0.31 / 0.27	0.95 (0.65-1.40)	0.79 (0.48-1.31)	0.65 (0.34-1.23)	1.10 (0.49-2.48)
p trend		0.88	0.50	0.17	0.52
Continuous per 0.1 mg/day		0.99 (0.83-1.19)	0.95 (0.73-1.23)	0.88 (0.62-1.23)	1.05 (0.71-1.53)
Supplement use					
No		1 (reference)	1 (reference)	1 (reference)	1 (reference)
Supplemental vitamin C user		0.95 (0.57-1.58)	1.07 (0.57-2.02)	0.64 (0.23-1.75)	1.76 (0.79-3.95)
Supplemental vitamin E user		1.83 (0.89-3.78)	1.42 (0.50-4.01)	1.92 (0.59-6.31)	0.81 (0.11-6.07)
Supplemental vitamin AD user		1.42 (0.74-2.73)	1.04 (0.41-2.67)	1.12 (0.33-3.74)	0.95 (0.22-5.75)
Supplemental multivitamin use		1.51 (0.91-2.51)	1.43 (0.73-2.79)	0.72 (0.22-2.28)	2.51 (1.10-5.75)

All models adjusted for age, sex, smoking (current smoker yes or no; number of cigarettes per day; number of smoking years), BMI and history of hypertension. Supplemental vitamin C, E, AD and multivitamin use are simultaneously entered in one model.

We also investigated possible interaction by sex and cigarette smoking status. Although we observed a statistically significant interaction of sex and beta-carotene intake for RCC (p=0.03), stratified analyses showed that there was no consistent differential effect for sex over the quintiles (results not shown). Table 7.3 shows results stratified by smoking and p values for interaction with smoking. There were no statistically significant differential results for the intake of carotenoids, vitamins or the use of supplements. We repeated these analyses for the subgroups of clear-cell cases with or without a *VHL* gene mutation. No conclusions could be drawn based on these results since the low number of cases, especially in the supplemental vitamin use groups, hampered analyses (data not shown).

Table 7.3 Relative risks for the association of carotenoid and vitamin intake and renal cell carcinoma (RCC), stratified by smoking status. Netherlands cohort study on diet and cancer 1986-1997.

Carotenoid/Vitamin	Never smokers RR (95% CI)	Ex-smokers RR (95% CI)	Current smokers RR (95% CI)	p value for interaction
Number of cases	75	111	98	
Number person-years in subcohort	16,543	15,182	11,247	
Alpha-carotene				
Q1	1 (reference)	1 (reference)	1 (reference)	0.29
Q2	0.54 (0.25-1.13)	1.16 (0.65-2.09)	0.80 (0.41-1.58)	
Q3	0.80 (0.41-1.56)	0.74 (0.39-1.41)	0.93 (0.48-1.80)	
Q4	0.63 (0.30-1.29)	0.66 (0.35-1.26)	1.05 (0.55-2.01)	
Q5	0.46 (0.22-0.96)	1.06 (0.58-1.95)	1.22 (0.65-2.30)	
p trend	0.09	0.53	0.39	
Continuous per 0.1 mg/day	0.95 (0.91-0.99)	1.00 (0.96-1.03)	1.01 (0.98-1.05)	
Beta-carotene				
Q1	1 (reference)	1 (reference)	1 (reference)	0.18
Q2	0.80 (0.39-1.66)	0.54 (0.28-1.02)	1.02 (0.52-2.01)	
Q3	0.84 (0.40-1.74)	0.80 (0.45-1.45)	0.88 (0.43-1.79)	
Q4	0.99 (0.49-1.99)	0.48 (0.26-0.92)	1.30 (0.67-2.51)	
Q5	0.53 (0.24-1.17)	0.96 (0.54-1.68)	1.47 (0.77-2.78)	
p trend	0.25	0.80	0.18	
Continuous per 1 mg/day	0.86 (0.74-1.00)	1.00 (0.87-1.13)	1.06 (0.94-1.19)	
Lutein+Zeaxanthin				
Q1	1 (reference)	1 (reference)	1 (reference)	0.57
Q2	0.53 (0.24-1.18)	0.76 (0.39-1.46)	0.67 (0.35-1.30)	
Q3	0.83 (0.41-1.67)	1.02 (0.55-1.87)	0.53 (0.26-1.08)	
Q4	0.96 (0.49-1.88)	1.09 (0.59-2.00)	0.75 (0.39-1.42)	
Q5	0.58 (0.27-1.22)	0.92 (0.49-1.75)	1.13 (0.64-1.99)	
p trend	0.49	0.78	0.61	
Continuous per 1 mg/day	0.91 (0.73-1.15)	1.04 (0.87-1.24)	1.04 (0.90-1.20)	

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Carotenoid/Vitamin	Never smokers RR (95% CI)	Ex-smokers RR (95% CI)	Current smokers RR (95% CI)	*p* value for interaction
Number of cases	75	111	98	
Number person-years in subcohort	16,543	15,182	11,247	
Beta-cryptoxanthin				
Q1	1 (reference)	1 (reference)	1 (reference)	0.24
Q2	0.59 (0.26-1.36)	1.06 (0.54-2.07)	1.56 (0.81-3.02)	
Q3	0.91 (0.43-1.93)	0.79 (0.39-1.60)	1.27 (0.63-2.57)	
Q4	1.26 (0.63-2.52)	1.21 (0.64-2.29)	2.30 (1.23-4.31)	
Q5	0.82 (0.38-1.76)	1.52 (0.82-2.81)	0.97 (0.46-2.07)	
p trend	0.65	0.14	0.42	
Continuous per 0.05 mg/day	1.01 (0.95-1.07)	1.06 (1.01-1.11)	1.02 (0.96-1.08)	
Lycopene				
Q1	1 (reference)	1 (reference)	1 (reference)	0.81
Q2	0.81 (0.37-1.77)	1.10 (0.54-2.24)	0.82 (0.41-1.65)	
Q3	1.21 (0.59-2.47)	1.28 (0.65-2.54)	0.92 (0.47-1.83)	
Q4	0.86 (0.40-1.86)	1.83 (0.96-3.49)	1.42 (0.76-2.63)	
Q5	1.25 (0.61-2.54)	1.44 (0.73-2.84)	0.92 (0.47-1.81)	
p trend	0.54	0.09	0.60	
Continuous per 0.5 mg/day	0.98 (0.80-1.22)	1.06 (0.85-1.32)	0.97 (0.73-1.29)	
Vitamin A (retinol)				
Q1	1 (reference)	1 (reference)	1 (reference)	0.38
Q2	0.85 (0.39-1.83)	1.54 (0.86-2.77)	0.67 (0.34-1.34)	
Q3	1.31 (0.66-2.60)	1.02 (0.54-1.94)	0.76 (0.38-1.50)	
Q4	0.81 (0.37-1.75)	0.80 (0.40-1.60)	0.62 (0.31-1.24)	
Q5	0.84 (0.39-1.81)	1.28 (0.69-2.38)	1.19 (0.64-2.20)	
p trend	0.65	0.83	0.55	
Continuous per 0.1 mg/day	0.97 (0.90-1.04)	1.01 (0.95-1.07)	1.00 (0.96-1.04)	
Vitamin C				
Q1	1 (reference)	1 (reference)	1 (reference)	0.29
Q2 + Q3	0.97 (0.50-1.87)	0.81 (0.47-1.42)	1.62 (0.91-2.89)	
Q4 + Q5	0.99 (0.52-1.89)	0.98 (0.57-1.68)	1.04 (0.57-1.90)	
p trend	0.99	0.85	0.78	
Continuous per 10 mg/day	0.98 (0.93-1.03)	1.03 (0.98-1.08)	1.01 (0.97-1.05)	
Vitamin E				
Q1	1 (reference)	1 (reference)	1 (reference)	0.25
Q2	0.67 (0.32-1.40)	0.69 (0.35-1.36)	0.98 (0.48-2.01)	
Q3	0.97 (0.49-1.91)	1.13 (0.61-2.10)	1.15 (0.59-2.21)	
Q4	0.75 (0.37-1.51)	1.32 (0.73-2.39)	1.00 (0.50-1.98)	
Q5	0.49 (0.22-1.07)	0.94 (0.50-1.75)	1.72 (0.91-3.28)	
p trend	0.13	0.45	0.14	
Continuous per 5 mg/day	0.88 (0.69-1.12)	1.01 (0.88-1.15)	1.19 (1.02-1.40)	

►

Carotenoid/Vitamin	Never smokers RR (95% CI)	Ex-smokers RR (95% CI)	Current smokers RR (95% CI)	*p* value for interaction
Number of cases	75	111	98	
Number person-years in subcohort	16,543	15,182	11,247	
Folate				
Q1	1 (reference)	1 (reference)	1 (reference)	0.17
Q2	0.92 (0.43-1.94)	0.66 (0.33-1.33)	0.97 (0.51-1.86)	
Q3	1.56 (0.80-3.03)	1.05 (0.57-1.94)	0.71 (0.37-1.37)	
Q4	0.66 (0.29-1.51)	1.25 (0.68-2.29)	0.62 (0.31-1.24)	
Q5	0.79 (0.36-1.74)	1.05 (0.55-1.98)	0.96 (0.52-1.80)	
p trend	0.40	0.32	0.59	
Continuous per 0.1 mg/day	0.85 (0.58-1.23)	1.24 (0.91-1.69)	0.87 (0.65-1.15)	
Supplement use				
No	1 (reference)	1 (reference)	1 (reference)	
Supplemental vitamin C user	0.61 (0.17-2.15)	1.05 (0.49-2.27)	1.09 (0.46-2.56)	0.69
Supplemental vitamin E user	1.76 (0.38-8.08)	2.22 (0.83-5.95)	1.13 (0.22-5.86)	0.85
Supplemental vitamin AD user	1.23 (0.36-4.19)	0.89 (0.26-3.07)	2.63 (0.87-7.94)	0.40
Supplemental multivitamin use	1.16 (0.40-3.34)	2.02 (0.97-4.21)	1.41 (0.52-3.87)	0.71

Models adjusted for age, sex, BMI, history of hypertension, and for ex and current smokers additionally adjusted for: number of cigarettes per day; number of smoking years. Supplemental vitamin C, E, AD and multivitamin use are simultaneously entered in one model.

Discussion

We investigated the association of several dietary carotenoids and vitamins (A, C, E and folate) and supplemental vitamin C, E, AD and multivitamin use and RCC incidence in the Netherlands Cohort Study on diet and cancer (NLCS). We observed no association for carotenoids and vitamins from diet and RCC risk. This is not surprising, since we also observed null associations for vegetable and fruit consumption and RCC risk[37]. Contrary to expectations, we observed a small statistically non-significant increased risk with supplemental vitamin E, AD, and multivitamin use.

Important strengths from the NLCS are that exposure was assessed before the diagnosis of cancer and that only incident cases were included. Therefore, recall bias is not likely to have influenced our results. Furthermore, selection bias is also unlikely because of the high completeness of follow-up of cases and subcohort members.

The consumption of vegetables and fruits, which contribute the most to the intake of carotenoids and vitamins, was extensively measured in the NLCS, using a validated semi-quantitative food-frequency questionnaire. Misclassification of exposure might have occurred. However, from our validation study it was concluded that the questionnaire could satisfactorily rank subjects according to the intake of nutrients and food groups[26]. In a reproducibility study, it was further demonstrated that the single food frequency questionnaire measurement could characterize dietary habits for a period of at least 5 years[38]. If misclassification has occurred, we expect this to be

nondifferential and risk estimates are most likely biased towards the null value. This may explain our negative results. However, for vegetable and fruit consumption, a very important source of carotenoids and vitamins, we believed this to be an unlikely explanation for the observed null association with RCC[37], since statistically significant associations had been observed for vegetable and fruit consumption and lung[39] and colorectal[40] cancer within the NLCS previously . However, this was less clear for previous studies on carotenoids and vitamins within the NLCS[41-44]. The assessment of intake of vitamin supplements was also shown to be reasonably good in our study[32].

The number of cases in this prospective cohort study, the second to report on carotenoids, vitamins and supplement intake, was reasonably large. The Iowa Women's Health Study[13] was the first cohort study to report on carotenoids, vitamins and supplement use, analyzing 124 incident cases after 15 years of follow-up. In the current study, we observed 314 incident RCC cases after 11.3 years of follow-up.

We cannot exclude the possibility that residual confounding has influenced our results, even though we tried to investigate and adjust for the appropriate confounding factors. All possible confounding factors as reported from the literature and measured within the cohort were included in the analyses if associated with RCC risk and correlated with at least one of the exposure factors in our population. Chance may have played a role since a large number of associations was tested, but we hardly observed statistically significant associations. Finally, power may have been a problem since the number of cases in subgroup analyses was sometimes low, although results across quintiles did not point in the direction of a dose-response relationship.

For carotenoids, there was an indication for a u-shaped association with RCC. This was less clear in the case groups of clear-cell RCC, and clear-cell RCC with or without a *VHL* gene mutation, possibly because of the smaller number of cases. This observation is in line with the observation of a u-shaped association with higher mutation frequencies at lower and higher intakes of dietary carotenoid intake[10]. Despite these suggestive results, we only observed a statistically significant inverse association in quintile 2 of lutein+zeaxanthin intake. For lycopene and beta-cryptoxanthin, risks seemed to be increased, without an indication of a u-shaped association. Lycopene is mainly derived from tomatoes, and citrus fruits including mandarins are an important source of beta-cryptoxanthin. Both tomato consumption and mandarin consumption were associated with a increased RCC risk in our population[37]. We could not explain the previously observed results for tomato and mandarin consumption and ascribed them to chance. Most results for carotenoids were not statistically significant, leading us to conclude that there was no association with RCC risk. These results are in line with most other studies, which reported statistically non-significant inverse and null associations[13,16,20,21]. The only study to report statistically significant inverse associations for alpha-carotene, beta-carotene, lutein, and beta-cryptoxanthin was a large population based case-control study including 1,204 cases[15].

We observed no association of dietary vitamin intake and RCC risk. To our knowledge, folate has not been investigated in relation to RCC risk before. For vitamins A and E

null associations and non-statistically significant inverse associations have been reported[13,15,16,21]. For vitamin C intake, reported associations were also not statistically significant, but generally somewhat more suggestive of a possible protective effect[13,15,16,20].

Supplemental vitamin E and multivitamin use seemed associated with an increased RCC risk (not statistically significant). One trial on vitamin supplementation and RCC risk was carried out (the ATBC study[12]). In this trial, male smokers were randomly assigned to supplemental alpha-tocopherol, beta-carotene, alpha-tocopherol *and* beta-carotene, or a placebo. No statistically significant differences in incidence/mortality rates of RCC were observed[12]. However, an inverse association was observed with multivitamin supplement use in the Iowa women's health study (RR: 0.63; 95% CI: 0.42-0.93)[13]. Two other large population-based case-control studies reported a null association with supplement use[15,16].

We also investigated possible effect-modification by smoking. We did not observe a differential effect for never, ex and current smokers. Thus far, stronger inverse association for non-smokers[16] as well as non-differential effects[15] have been published. In the study by Hemminki *et al.*[8] it was noted that smoking appeared to change the outcome of many variables (although smoking itself was not a risk factor for *VHL* gene mutations). However, small numbers in that study did not allow for testing of smoking as a true effect modifier. In the current study, numbers were also too small to draw firm conclusions from interaction tests and from analyses, stratified by smoking.

We were able to investigate risk factors for the specific histological subtype clear-cell RCC and also for the presence of *VHL* gene mutations, which has not been done before for vitamins and carotenoids. There has only been one report on diet and *VHL* gene mutations based on a case-only analysis[8]. The authors of that study concluded that their results provide evidence that the intake of vegetables, selenium and particularly citrus fruit protects the renal *VHL* gene from mutational insults, although chance results could not be ruled out in that relatively small study[8]. Based on case-only comparisons, we would have observed a non-statistically significant protective effect of supplemental multivitamin intake on *VHL* gene mutations (OR: 0.22; 95% CI: 0.05-1.02). However, compared to the subcohort, the risk for wildtype *VHL* tumors was increased (RR: 2.51; 95% CI: 1.10-5.75) which shows that case-only comparisons can be misleading. Thus, we observed an increased risk for *VHL* wildtype clear-cell tumors for supplement use and not a protective effect of supplemental multivitamin use on *VHL* gene mutations.

In summary, no statistically significant dose-response associations were observed. Stratified results by *VHL* gene mutation showed no statistically significant differential effects, although results were sometimes suggestive of a differential effect. These associations should be investigated by others to confirm the current observations.

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8

General discussion

Classical epidemiological studies have generally reported weak associations between most risk factors and renal cell carcinoma (RCC); moreover, results of studies on the same risk factors were often equivocal. One way to obtain stronger associations is to reduce heterogeneity at the level of the endpoint. Therefore, in addition to studying the association between risk factors and RCC risk in a classical epidemiological approach, we investigated risk factors in relation to the presence of specific gene mutations, as a more specific endpoint, in a molecular-epidemiological approach. Results from these analyses could ultimately substantiate or lead to hypotheses on the etiology of RCC. Mutations in the *von Hippel-Lindau (VHL)* gene were chosen as a more specific endpoint since *VHL* gene mutations are rate-limiting events in the carcinogenesis of clear-cell RCC[1,2] and it is the most common genetic aberration. In this chapter the main findings described in this thesis, strengths and limitations, implications of analyses including *VHL* gene mutations, conclusions and suggestions for further research are discussed.

Main findings

The relationship between clinical and pathological parameters and the *VHL* gene mutational status was investigated. Approximately 60% of all clear-cell tumors presented with one or more *VHL* gene mutations. Some heterogeneity was present, i.e. in some instances two mutations were observed in one paraffin block, or one paraffin block from a tumor showed no mutations while we did find a mutation in another paraffin block from the same tumor. Regarding clinical parameters, only tumor size appeared to differ by *VHL* gene mutational status, with larger tumors in case of at least one *VHL* gene mutation, although this was not statistically significant.

Height was associated with a statistically significantly increased RCC risk only in women. Body mass index and gain in body mass index since age 20 were associated with a statistically significantly increased risk of RCC for men and women. We furthermore assessed whether energy intake and/or physical activity were independent risk factors and whether these factors explained the association between body mass index and RCC. Energy intake was not related to RCC risk, while the association between physical activity and RCC remained unclear. Energy intake and physical activity did not explain the association between body mass index and RCC risk.

Vegetable and fruit consumption were not associated with RCC risk. Most specific botanical groups and individual vegetables and fruits were also not associated with RCC risk. In addition, no association between carotenoid and vitamin intake and RCC risk was observed. Only supplemental vitamin intake appeared to be associated with a slightly increased risk. Stratified analyses based on mutational status showed somewhat higher risks in the group of *VHL* wildtype tumors (tumors without a mutations in the *VHL* gene) for alpha-carotene, beta-cryptoxanthin, folate, and vitamin C and multivitamin supplement intake. Cigarette smoking was no effect modifier for the association between vegetable and fruit consumption and RCC risk or for the

association between carotenoid and vitamin intake and RCC risk, or risk of *VHL* mutated or *VHL* wildtype tumors.
Cigarette smoking itself was associated with an increased RCC risk, especially for men. This is probably the result of a difference in smoking habits between men and women. Risks increased with increasing frequency of cigarettes smoked per day and increasing number of smoking years for men, while the number of cases did not allow us to investigate this for women. We did not find an association of cigarette smoking and specific *VHL* gene mutations. Risks were the same or slightly higher for wildtype tumors compared to *VHL* mutated tumors, suggesting cigarette smoking may not cause RCC through *VHL* gene mutations.
Both hypertension and antihypertensive medication use were associated with a non-statistically significant increased risk of RCC. Stratified analyses based on the *VHL* gene mutational status showed a somewhat higher risk in the group of tumors with a *VHL* gene mutation for a history of hypertension, while for antihypertensive medication use the risk appeared to be higher in the group of *VHL* wildtype tumors, suggesting different pathways.

Strengths and limitations

Both classical epidemiological and molecular epidemiological studies may suffer from different types of bias and confounding. Compared to the classical epidemiological studies, molecular epidemiological studies include molecular data, therefore some additional issues are specifically important, e.g. misclassification of the molecular data. In the following sections, the most important general methodological issues and methodological issues specific to molecular epidemiological studies will be discussed.

General methodological issues

Measurement of independent variables
Recall bias is unlikely in this study since the self-administered questionnaire was filled out before cancer was ascertained (all prevalent cancers were excluded from analyses). There are two possible issues that deserve further attention; misclassification of exposure and latency.
First, some misclassification of exposure may have occurred, since exposure was assessed using a self-administered questionnaire. In most instances the effect of the possible presence of this phenomenon could be argued. Height, weight, weight at age 20, a history of hypertension, antihypertensive medication use and cigarette smoking were all self-reported, as were all other risk factors in this study. This may lead to under- or over-reporting. However, these factors were measured before the diagnosis of RCC and therefore misclassification is likely to be non-differential with respect to the outcome. Generally, non-differential misclassification leads to attenuated rate ratios[3]. However, in case of variables with more than 2 categories (such as physical activity),

the result of possible misclassification of exposure is difficult to predict, and may be away from the null for one of the categories[3]. Another possible source of misclassification of exposure is changing habits. In this study population dietary habits were observed to be relatively stable during at least 5 years[4].
Second, this study was based on 55-69 year olds at baseline because dietary habits (and their contrasts) are stabilized, and such a cohort will yield sufficient cases for meaningful analyses within a reasonable time period[5]. Exposure factors should be measured at a relevant time frame and latency estimates could help determine this timeframe. However, there is uncertainty concerning latency and estimates for latency are seldom reported, because they are difficult to establish. To our knowledge, few studies have reported estimates for latency periods; latency periods are usually estimated at decades e.g. one study estimated latency for smoking and lung cancer at 30 years based on population figures[6]. For RCC, estimates for latency exist only for specific occupational exposures as measured in occupational cohorts. The question then arises whether the incident cancer can be attributed to the investigated factors. It has to be acknowledged that some factors may not be stable or the latency period may not have been adequate, which could have resulted in misclassification and erroneous results. However, most dietary factors appear to be relatively stable in an elderly cohort for a period of at least five years and perhaps even for a decade[4].

Follow-up

Loss to follow-up is the primary source of potential selection bias in prospective cohort studies (provided it is differential across determinant strata)[5,7,8]. To minimize the possibility of selection bias, the study area and population in the present cohort study were chosen in a way to ensure sufficient follow-up coverage[9]. The completeness of incident cancer follow-up was estimated to be more than 96%[9]. The subcohort has been followed up for vital status information biennially by mail, and in case of non-response completed by contacting municipal population registries. Only two male subcohort members were lost to follow-up after 11.3 years of follow-up. It is unlikely that our results are influenced by selection bias because of the high completeness of incident cancer and of person-years follow-up.

Methodological issues specific to molecular epidemiological studies

Selection bias resulting from tissue collection

Paraffin block collection to complete information on molecular data for all cases is of great importance in this study. If the collected paraffin blocks are a selection of the blocks in the entire population, observed results may only apply to the investigated population and not to the entire population. Specifically, the absence of tumor tissue, if differential, may lead to selection bias[10].

Generally, there are three reasons why tumor tissue may not be available[10]:

1. In some instances, no material is collected as part of the diagnostic process, e.g. if only low-grade tumors are excised: Overall, a high percentage (78%) of patients diagnosed with RCC in the region of the Netherlands covered by the IKL (which is representative for the Netherlands) is treated surgically, although the percentage of surgical procedures was somewhat lower for patients with stage IV (59%) or stage X (not assessable) (38%) tumors (personal communication, IKL, the Netherlands). If this occurred, no histology report or unsuitable samples for our study (e.g. biopsy material only, see also reason 3) would be present. The absence of a histology report in this study may, however, also be the result of record linkage errors or of the incompleteness of PALGA[9,11]. In our study, histology reports, including information on which pathology laboratory holds the paraffin material, were present for most patients (273/337=81%).
2. Tissue samples may have been unavailable because the tissue was lost or destroyed or the hospital would not release the sample:
   Great effort was undertaken to retrieve tumor material from all laboratories with relevant material. Most laboratories were visited to collect paraffin blocks, although some laboratories preferred to mail us the requested paraffin blocks. In the current study, only three pathology laboratories refused to release material (blocks from 10 cases) and for 3 cases material could not be located in the laboratory.
3. The sample preparation is inappropriate for the molecular analysis (e.g. sample is too small, too degraded to assay, lacks normal DNA, or sample preservation method is incompatible with the molecular analytical protocol):
   Twenty-four samples were not usable for this study because they were too small, too degraded, or lacked tumor material.

There were no large differences in investigated exposure variables and possible confounding factors between cases with collected paraffin material (N=235) and cases without collected paraffin material (N=102). Only the percentage of antihypertensive medication use was different between cases with paraffin blocks collected and those without paraffin blocks (26.8% versus 14.7%, respectively). This difference can probably be ascribed to chance since many variables were tested and none of these variables, including a history of hypertension, showed a statistically significant difference between the groups with and without collected paraffin material. The fact that we were able to retrieve a high proportion of samples, the reported reasons for not being able to collect paraffin material and the absence of differences between cases with or without paraffin material led us to conclude that our results are not likely to be affected by selection bias.

Ascertainment of endpoint: histological classification

One experienced pathologist revised all slides, resulting in a consistent classification of histology according to the World Health Organization (WHO) classification of Tumours of 2002[12], which is a strength of this study. This information on histology

could not be extracted from the records of the Netherlands cancer registry or PALGA. The classification of RCC is relatively new and until recently most parenchymal kidney cancers were classified as Grawitz tumors. Most studies on etiological factors for RCC did not incorporate information on the specific histological subtype, while some etiological factors may be linked more closely to certain histological subtypes, as is the case for e.g. smoking and lung cancer[13].

Ascertainment of endpoint: mutational status

For the determination of the mutational status of the tumors, a large number of PCR gene based diagnostic tests had to be carried out. We undertook a pilot study to assess PCR-SSCP as a screening tool preceding direct sequencing (chapter 4). In this pilot study, no mutations were missed in the SSCP compared to direct sequencing, and we estimated the upper 95 confidence limit for the chance of missing mutations as a result of using PCR-SSCP as a screening instrument at between 3.7% and 16.9%. These results led to the decision to use PCR-SSCP as a screening tool. We used 6 primer pairs to cover the *VHL* gene, which means that for every sample at least 6 PCRs were performed, followed by SSCP analyses. The percentage of observed mutations (61%) was high in comparison to other studies, which led us to believe that we did not miss large numbers of mutations. We interpreted all observed mutations to be "true" mutations, when the mutations were confirmed in two independent experiments starting from DNA isolated from the paraffin block: PCR-SSCP and direct sequencing.
Surprisingly, discordant cases were found; we observed different mutations in the same paraffin block (13 cases) or the absence versus the presence of a mutation in different paraffin blocks (10 cases) obtained from the same tumor. These results might indicate tumor heterogeneity, while in line with the general opinion clonal *VHL* mutations were expected. However, others have also reported multiple mutations per primary tumor[14-16]. This observation may be the result of increasing genetic instability leading to additional mutational events in tumor development. All in all, we believe that we obtained valid results for a large population-based study that can be used to investigate the association between risk factors and clear-cell RCC with or without a *VHL* gene mutation. However, we are aware of the fact that we investigated the association of risk factors to prevalent and not incident mutations. As a result, we could not determine whether an observed association to a mutation is important in the initiation or promotion phase of tumor development.

Genotype versus phenotype

The VHL protein (pVHL) and not the *VHL* gene is eventually important for the tumor suppressing effect. One may argue that the classification into wildtype versus mutated should be made at the protein level (phenotype) and not at the DNA level (genotype), as was done in the current study. Most observed mutations lead to an altered and/or inactive protein. However, this is not the case for silent mutations (N=13). Also, a mutation in the first 54 codons (N=6) may not cause the loss of tumor suppressor

function of the protein, although other functions may be affected. Furthermore, for missense mutations the effect on the protein or on the protein function may not always be clear. This would lead to misclassification at the level of the presence of functional protein, resulting in an underestimation of heterogeneity between samples classified as *VHL*-mutated or wildtype. However, given the study design and material and methods available, we were only able to investigate the presence of *VHL* gene mutations and not the presence of (functional) protein. Immunohistochemical staining for pVHL is possible, but the presence of staining is not related to the deletion of 3p gene sequences or *VHL* mutations[17,18]. Even though absence of functional pVHL is required to lose tumor suppressor function, in molecular carcinogenesis not directly the function but the mutation and type of mutation are of primary interest.

Statistical analyses

Using a more specific endpoint by definition reduces the number of cases in the analyses. This lack of power may have led to the absence of statistically significant associations, but if a differential association between risk factors and mutational status exists, we still would have expected to find indications for a consistent difference in point estimates based on mutational status.

Investigating heterogeneity of mutational status can be carried out by investigating different case groups (such as cases with a *VHL* gene mutations or cases without a *VHL* gene mutation) in comparison to a "control group" or by comparing these different case groups to each other (case-only analyses). The case-only approach is commonly used in molecular-epidemiological studies[19-22]. However, using merely a case-only design is not preferable since no comparison to the population can be made. Therefore, results of a case-only design can only lead to conclusions on the possible existence of mutational heterogeneity, and if a protective effect for mutations is observed, this does not necessarily mean that the risk of cancer is also reduced. This can be illustrated using the data on supplemental vitamin use from chapter 7. Based on case-only comparisons, a nearly significant protective effect of multivitamin supplement intake on *VHL* gene mutations was observed (OR: 0.22; 95% CI: 0.05-1.02). However, compared to the subcohort, the risk for wildtype *VHL* tumors was increased (RR: 2.51; 95% CI: 1.10-5.75), while the risk for *VHL* gene mutated tumors equaled 0.72 (95% CI: 0.22-2.28). This shows that the inclusion of a population-based control group in this type of research to show the relation to the population is preferable.

Implications of analyses including *VHL* gene mutations

We believed that this molecular epidemiological study could elucidate part of the black box between exposure to risk factors and the not very specific endpoint of RCC. Thus far, however, associations between risk factors and molecular endpoints have not led to more convincing conclusions or clear differential effects. Even for cigarette smoking, we did not find differential results, while cigarette smoking has clearly been shown to

cause mutations in human DNA, also in tissues not directly exposed to tobacco smoke[23]. Cigarette smoke metabolites have been detected in urine[24], suggesting exposure of the renal parenchyma to mutagenic cigarette smoke constituents or metabolites. Furthermore, N-nitrosodimethylamine induced tumors were associated with *VHL* gene mutations (predominantly G:C→A:T transitions) in rats[25]. Unfortunately, the numbers in our analyses were too small to draw conclusions on specific mutations, as well as on the association of the number of cigarettes smoked per day and the number of smoking years and the *VHL* gene mutational status. This could have given us a more detailed picture of cigarette smoking in relation to the presence of *VHL* gene mutations. There are also other factors that may explain of the absence of a stronger association between cigarette smoking and the presence of *VHL* gene mutations. We know that not all persons exposed to cigarette smoke develop cancer, probably as a result of differences in the individual genetic profile of metabolizing enzymes resulting in differences in mutagenicity. Also, other risk factors, besides cigarette smoking may be involved. This may not just be one (unknown) factor but a (unknown) mechanism, reflected in a combination of factors. We may speculate on lipid peroxidation, since this seems to be associated with many risk factors for RCC, e.g. with a high relative body weight, essential hypertension, cigarette smoking and low vitamin E intake. Byproducts of lipid peroxidation have been shown to react with renal DNA to form adducts which may ultimately lead to DNA mutations, and increased lipid peroxidation was reported to occur more extensively in primary renal cell carcinomas than in surrounding cancer-free tissue[26]. Another possibility may be that cigarette smoking is not implicated in the initiation phase of tumor development, in which presumably *VHL* gene mutations occur, but in the promotion phase, affecting other (unknown) targets.

The association with hypertension was stronger in RCC patients with *VHL* gene mutations, while the association between diuretic use and *VHL* wildtype tumors was stronger, implying the possibility of different pathways for a history of hypertension and diuretic use. Hypertension is associated with increased lipid peroxidation[26]. Diuretic therapy, specifically hydrochlorothiazide use, might be carcinogenic through conversion in the stomach to carcinogenic nitroso-derivatives or through a low-grade carcinogenic effect on the renal tubular cell, its principal target[27].

Results were suggestive of higher rate ratios for alpha-carotene, beta-cryptoxanthin, folate and supplemental vitamin C and multivitamin intake for wildtype *VHL* tumors. In a study by Nyberg *et al.*, diet was linked to somatic mutations in vivo[28]. The mutant frequency was significantly decreased with higher vitamin C intake, while a U-shaped association between mutant frequency and dietary carotenoid intake was observed[28]. Carotenoid and vitamin intake may only be beneficial in case of oxidative stress, e.g. as a result of cigarette smoking[28]. Unfortunately the numbers in our analyses did not allow us to draw conclusions on analyses stratified by smoking status in *VHL* mutated and wildtype groups, even though cigarette smoking itself did not seem to be associated with *VHL* gene mutations. For the total group of RCC cases, we did not observe interaction between carotenoids and vitamins and smoking.

In general, there is another likely reason for not finding associations between risk factors and *VHL* gene mutations. Only about 60% of clear-cell RCC cases present with a *VHL* gene mutation, while the other 40% also developed clear-cell RCC, indicating that there have to be other genes or mechanisms that play a role in clear-cell RCC carcinogenesis. For example, repair processes have to fail for a mutation to be sustained and apoptotic mechanisms have to be disabled for a mutated cell to survive. A stepwise accumulation of mutations has been shown to be necessary in e.g. the evolution of colon cancer, which requires biallelic loss of function of several tumor suppressor genes and gain of function of one or more oncogenes[29]. Similarly, to develop clear-cell RCC, more than the two hits to disable both alleles of the *VHL* gene may be necessary. The investigated model of one or more exposure factors leading to or protecting against *VHL* gene mutations leading to clear-cell RCC may have been too simplistic.

Conclusions

In this study we confirmed the positive association of BMI, cigarette smoking, a history of hypertension and antihypertensive medication use with RCC risk. We observed no association with vegetable and fruit consumption and carotenoid and vitamin intake. By specifying the endpoint, based on histology and the presence of *VHL* gene mutations, we hoped to gain insight into the etiology of RCC. We did not observe clear differential results based on the presence of *VHL* gene mutations, suggesting that the investigated risk factors may be associated with other genes in the same pathway of *VHL* or with other pathways. Consistency over studies could not be shown yet, since this is only the third study that included *VHL* gene mutations as an endpoint, and the three studies are difficult to compare because of differences in design and risk factors studied.

Recommendations for future research

In future studies, the association between other risk factors such as occupational factors and hormonal factors, and RCC and specifically to *VHL* gene mutations should be investigated. Occupational exposures may directly cause mutations, while risk factors, such as obesity, a history of oophorectomy, and diabetes, may induce lipid peroxidation, which may ultimately cause mutations[26]. Stratified analyses based on a combined pattern of risk factors, e.g. comparing a group of persons with high expected lipid peroxidation to a group with low expected lipid peroxidation based on their risk factor profile, seems feasible and may lead to new insights.

There are indications that exposures early in life may be particularly relevant in carcinogenesis. It has been shown that a low caloric intake in childhood has been associated with reduced cancer risk (other than cancer related to smoking)[30,31], although not consistently[32,33]. Early life exposures should be investigated when possible.

The numbers in our study did not allow for strong conclusions when incorporating *VHL* gene mutations as a more specific endpoint. Confirmation of our results by other large studies and pooling of existing studies with information on risk factors and *VHL* gene mutations could lead to more clarity regarding associations between risk factors and *VHL* gene mutations.

We were not able to investigate the methylation status in this study, yet. This should be further investigated, since samples with hypermethylation of a normally unmethylated promoter region would have been classified as wildtype in our analyses, while this renders the *VHL* gene transcriptionally inactive. The association between risk factors affecting the presence of methyl donors, such as alcohol consumption and folate intake, and hypermethylation should be investigated.

As discussed before, another gene or set of genes from the same pathway or other pathways may be involved in RCC. Hypoxia inducible factor (HIF) might be interesting, since this factor plays a central role in the hypoxia pathway. It has been shown that HIF is stabilized during hypoxia, in case of a mutated *VHL* gene (same pathway as under hypoxia), or as a result of receptor-mediated factors, such as growth factors, cytokines and circulatory factors[34]. These growth factors include insulin and insulin-like growth factor-1[35], factors that have been implicated in cancer development and progression as mitogens that play a pivotal role in regulating cell proliferation, differentiation, and apoptosis[36]. However, another report stated that hypoxia and insulin activate the same set of target genes downstream of HIF, such as vascular endothelial growth factor (VEGF), but that only hypoxia stabilizes HIF, while insulin does not[37]. Other types of genes to be considered for future investigations are genes involved in repair and apoptotic processes and newly discovered genes which appear to be relevant for RCC.

Finally, all persons handle carcinogenic substances differently. Polymorphisms in enzymes that metabolize substrates that are carcinogenic or become carcinogenic in the process of excretion are common[38]. The efficiency of these metabolizing enzymes determines the extent of exposure to a carcinogenic compound and is thus likely to affect cancer risk[38]. Therefore, it would be useful to take polymorphisms in metabolizing enzymes into account, e.g. by including information on CYP1A1 and GSTM1 as risk modifiers for the association between cigarette smoking and RCC risk.

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Summary

Kidney cancer is the ninth most common cancer in the European Union. Incidence and mortality rates are higher in developed regions and are approximately twice as high for men as for women. The 5-year survival is approximately 55%. Kidney cancer includes cancer of the renal parenchyma (renal cell carcinoma (RCC)) and cancer of the renal pelvis. These types of cancer differ with respect to anatomical location, type of cell from which these evolve (parenchymal cells versus urothelial cells, respectively) and their association with risk factors.

We confined our research to cancer of the renal parenchyma, RCC, which can be subdivided into several histological types. The most common histological subtype is clear-cell or conventional RCC, which accounts for approximately 75% of renal neoplasms in surgical series.

Aberrations in the *von Hippel-Lindau (VHL)* gene have been shown to be a common, early and distinct event in the development of clear-cell RCC. The *VHL* gene is a tumor suppressor gene, located on the short arm of chromosome 3 (3p25-26), and it is composed of three exons. Since the *VHL* gene is a tumor suppressor gene, both alleles need to be affected before the function will be lost. Biallelic *VHL* gene defects are observed in approximately 75% of sporadic clear-cell RCC. The human *VHL* gene encodes two VHL proteins: pVHL30 and pVHL19. Both proteins suppress tumor formation. Loss of pVHL creates an environment favorable for tumor growth.

The association of many risk factors with RCC risk has been investigated, but only cigarette smoking, obesity, a history of hypertension and use of diuretics were consistently linked to RCC risk. Although consistent, risks were not very high.

Generally, little attention is spent on the endpoint. However, RCC is a heterogeneous endpoint, i.e. there are several histological subtypes and sporadic mutations may be present or absent. Stratified analyses based on histology and the mutational status of a tumor may lead to additional insight in the carcinogenesis process.

In this thesis we investigated several hypotheses on the association between risk factors and the risk of RCC and in some instances with clear-cell RCC and the presence of mutations in the *VHL* gene within the setting of the Netherlands Cohort Study on diet and cancer (NLCS). The NLCS is a prospective cohort study, which started in September 1986. The cohort included 120,852 men and women, aged 55-69 years at the beginning of the study. All participants completed a self-administered questionnaire on dietary habits (food-frequency questionnaire), lifestyle, personal medical history, family history of cancer and demographic data. The study was designed as a case-cohort study, using all cases, while the person-time accumulated in the entire cohort was estimated using a random sample of 5,000 men and women (subcohort). Follow-up for incident cancer has been established by annual record linkage to the Netherlands cancer registry and PALGA, a national database of pathology records. After 11.3 years of follow-up, 337 incident histologically confirmed epithelial cancer cases were observed.

In chapter 2, we found BMI to be an independent risk factor for RCC (rate ratio (RR): 1.07; 95% CI: 1.02-1.12 per 1 kg/m^2 increment). A RR higher than 1 means that there

is an association with an increased risk (lower than 1: reduction of risk); the observed association is unlikely to be a chance result if the 95% CI does not include 1). Height in women (RR: 1.23; 95%CI: 1.02-1.46 per 5 cm increment) but not in men (RR: 0.97; 95% CI: 0.84-1.13 per 5 cm increment), and weight both in men (RR: 1.09; 95% CI: 0.98-1.20 per 5 kg increment) and women (RR: 1.11; 95% CI: 1.01-1.23 per 5 kg increment), also increased RCC risk. BMI gain since age 20 years increased the risk of RCC (RR: 1.06; 95% CI: 1.01-1.10). Energy intake was not associated with RCC, while the association of physical activity and RCC remains unclear. Furthermore, energy intake and physical activity did not explain the association of BMI and RCC.
We also observed that neither total vegetable and fruit consumption, nor the consumption of specific botanical groups of vegetables or fruits nor individual vegetables or fruits consumption were associated with a decreased RCC risk. Furthermore, there were no indications for a modifying effect by cigarette smoking, BMI or a history of hypertension (chapter 3).
To be able to investigate risk factors with the more specific endpoint of the presence of *von Hippel-Lindau* (*VHL*) gene mutations, we collected paraffin embedded tumor material from the large series of incident RCC cases in this cohort from 51 pathology laboratories, which has been described in chapter 4. Approval was obtained from the medical ethical committees of Maastricht University, PALGA, and the Netherlands cancer registry. We were able to identify the location of paraffin material for 273 cases out of the 337 incident cases. For 251 of 273 cases we collected paraffin blocks. After revision by a pathologist, tumor DNA from 235 cases was available for further analysis.
We observed at least one *VHL* gene mutation in 61% of the clear-cell RCC tumors; most were truncating mutations. No differences were observed in nuclear grade, TNM distribution or stage for tumors with or without a *VHL* gene mutation. *VHL* mutated tumors were on average 72.7 mm in size compared to a mean tumor size of 65.3 mm for wildtype *VHL* tumors. However, this difference was not statistically significant (chapter 4).
Cigarette smoking is a known risk factor for RCC. Cigarette smoke metabolites have been shown to cause mutations in human DNA, which is not restricted to tissues directly exposed to tobacco smoke. In chapter 5 we investigated the association of cigarette smoking to RCC and to *VHL* gene mutations in clear-cell RCC. For men, RRs for total RCC were 1.52 (95% CI: 0.89-2.59) and 2.07 (95% CI: 1.20-3.56) for ex- and current smokers compared to never smokers, respectively. Estimates for women equaled 0.95 (95% CI: 0.57-1.59) and 1.37 (95% CI 0.87-2.16), respectively. When stratified for *VHL* mutation status, RRs for current smokers compared to never smokers were 2.34 (95% CI: 0.79-6.94) and 2.95 (95% CI: 0.65-13.28) for *VHL* gene mutated and *VHL* wildtype tumors, respectively, for men. For women, these estimates were 0.82 (95% CI: 0.35-1.93) and 2.04 (95% CI: 0.94-4.45), respectively. Cigarette smoking was associated with RCC risk in men, but cigarette smoking was not specifically associated with *VHL* gene mutations, irrespective of sex. These results suggest that smoking may be associated with RCC risk, independent of *VHL* gene mutations.

A history of hypertension and use of antihypertensive medication have been associated to RCC risk, although, it remains unclear whether the increased risk is caused by hypertension or the medication. Hypertension (RR: 1.22; 95% CI: 0.94-1.58) and use of antihypertensive medication (RR: 1.14; 95% CI: 0.85-1.52) were associated with a slightly increased overall RCC risk in our study (chapter 6). A history of hypertension was associated with a non-significantly increased risk of clear-cell RCC with *VHL* gene mutations: (RR: 1.34; 95% CI: 0.87-2.07), and was not associated with the risk of clear-cell RCC without *VHL* gene mutations (RR: 0.88; 95% CI: 0.51-1.53). Use of diuretics (a type of antihypertensive) was not associated with *VHL* gene mutated clear-cell RCC (RR: 0.91; 95% CI: 0.45-1.81), but it was associated with clear-cell RCC without *VHL* gene mutations (RR: 2.11; 95% CI: 1.16-3.83). These results imply the possibility of different pathways for a history of hypertension and diuretic use, although false-positive findings as a result of multiple testing and small numbers cannot be excluded.

Dietary carotenoid and vitamin intake were not (inversely) associated with overall RCC risk (chapter 7). Supplemental vitamin E, AD and multivitamin use were associated with a non-statistically significantly increased risk. Results were suggestive of higher RRs for wildtype *VHL* tumors with alpha-carotene, beta-cryptoxanthin, folate and supplemental vitamin C and multivitamin intake.

Overall, in this study, we confirmed the positive association of BMI, cigarette smoking, a history of hypertension and antihypertensive medication use with RCC risk. We observed no association with vegetable and fruit consumption and carotenoid and vitamin intakes. We did not observe clear differential results between risk factors and the presence of *VHL* gene mutations, even though *VHL* gene mutations are an early, common and distinct feature of clear-cell RCC. One possible explanation may be that risk factors play a role in the promotion and not the initiation phase of renal carcinogenesis. Hypermethylation of the promoter area of the *VHL* gene may also render the *VHL* gene transcriptionally inactive, which should be further investigated. Furthermore, only about 60% of clear-cell RCC cases present with a *VHL* gene mutation. This indicates that there are other genes or mechanisms that have to play a role in clear-cell carcinogenesis, possibly similar to colon cancer, in which biallelic loss of function of several tumor suppressor genes and gain of function of one or more oncogenes is required.

Future research should investigate more risk factors and/or combine risk factors according to possible mechanisms. The associations investigated in this thesis should, however, be tested in other large studies. Pooling of our results with information on risk factors and *VHL* gene mutations from other studies may lead to more firm conclusions. Finally, other genes, such as genes involved in the angiogenesis pathway, genes that play a role in repair processes, or genes involved in apoptosis should also be considered for investigation in a molecular-epidemiological approach if there are reports on mutations or promoter hypermethylation in RCC.

Samenvatting

Nierkanker is het negende meest voorkomende type kanker in de Europese Unie. Incidentie en mortaliteitsratio's zijn hoger in de ontwikkelde landen en zijn ongeveer 2 keer zo hoog voor mannen dan vrouwen. De 5-jaarsoverleving bedraagt ongeveer 55%. Vaak wordt onder nierkanker niet alleen kanker van het nierparenchym, maar ook kanker van het nierbekken gerekend, maar kanker van het nierbekken is anders wat betreft anatomische locatie, het type cel waaruit de kanker zich ontwikkelt (urotheel cellen versus parenchym cellen) en de beschreven associaties met risicofactoren.
We hebben ons in ons onderzoek beperkt tot kanker van het parenchym, in het vervolg met nierkanker aangeduid, dat verder onderverdeeld kan worden in verschillende histologische subtypes. Het meest voorkomende histologisch subtype is heldercellige nierkanker, wat in ongeveer 75% van niertumoren in chirurgische series vastgesteld wordt.
Afwijkingen in het *von Hippel-Lindau (VHL)* gen zijn een vroege, veel voorkomende en karakteristieke gebeurtenis in de ontwikkeling van heldercellige niertumoren. Het *VHL* gen is een tumor suppressor gen, dat zich bevindt op de korte arm van chromosoom 3 (3p25-26), en omvat 3 exonen. In het geval van een tumor suppressor gen moeten beide allelen aangedaan zijn voordat de functie verloren gaat. Dit wordt gevonden in ongeveer 75% van de sporadische (niet erfelijke) heldercellige niertumoren. Mutaties leiden meestal tot een verkort, inactief eiwit. Het wildtype *VHL* gen kan twee eiwitten opleveren: pVHL30 en pVHL19. Beide eiwitten onderdrukken tumorgroei. Verlies van pVHL zorgt voor een omgeving die gunstig is voor tumorgroei.
De associatie tussen verschillende risicofactoren en het risico van nierkanker is al eerder onderzocht, maar alleen roken, overgewicht, hoge bloeddruk in de voorgeschiedenis en het gebruik van diuretica (medicijnen die voorgeschreven worden om de hoge bloeddruk te verlagen) werden consistent in verband gebracht met het risico van nierkanker. De gerapporteerde de risico's waren echter niet heel hoog.
Vaak wordt in epidemiologische analyses weinig aandacht geschonken aan de specificiteit van het eindpunt. Nierkanker is echter een heterogeen eindpunt omdat er verschillende histologische subtypen zijn, welke gekarakteriseerd kunnen zijn door de aan- of afwezigheid van specifieke mutaties. Gestratificeerde analyses gebaseerd op histologie en op de mutatie status van een tumor kunnen leiden tot nieuwe inzichten in het ontstaansproces van nierkanker.
In dit proefschrift hebben we verschillende hypothesen betreffende het verband van risicofactoren en de kans op nierkanker onderzocht. Indien gegevens ten aanzien van de aanwezigheid van mutaties in het *VHL* gen in heldercellige niertumoren op dat moment bekend waren, dan zijn deze ook geanalyseerd als een specifieker eindpunt. Dit onderzoek is uitgevoerd binnen de Nederlandse cohort studie naar voeding en kanker (NLCS). De NLCS is een prospectieve cohort studie, welke in september 1986 gestart is. In deze cohort studie zijn 120,852 mannen en vrouwen geïncludeerd die op dat moment 55–69 jaar oud waren. Alle deelnemers hebben een vragenlijst over voedingsgewoonten (voedselfrequentie vragenlijst), leefstijlfactoren, persoonlijke

medische geschiedenis, vragen over het voorkomen van kanker in de familie en demografische gegevens ingevuld. Het onderzoek is een zogenaamde case-cohort studie, waarin alle cases worden opgenomen die in het complete cohort ontstaan, terwijl de persoonstijd in het volledige cohort geschat word aan de hand van de opgebouwde persoonstijd in het subcohort (willekeurige steekproef) van 5,000 mannen en vrouwen. Incidente kanker werd vastgesteld door een jaarlijkse koppeling met de Nederlandse kankerregistratie en PALGA, een landelijke database van pathologie rapporten. Na 11.3 jaar waren er 337 incidente, histologisch bevestigde, epitheliale kanker.

In hoofdstuk 2 beschrijven we dat body mass index (BMI: gewicht in kilogram / lengte in meter^2) een onafhankelijke risicofactor is voor nierkanker (risico ratio (RR): 1.07; 95% betrouwbaarheidsinterval (BI): 1.02-1.12 per 1 kg/m^2 toename). Een RR groter dan 1 betekend dat er een verhoging van het risico is (kleiner dan 1: verlaging van het risico); indien het getal 1 niet binnen het 95% BI valt is het onwaarschijnlijk dat de gevonden associatie door toeval kan worden verklaard. Lengte voor vrouwen (RR: 1.23; 95% BI: 1.02-1.46 per 5 cm toename), maar niet voor mannen (RR: 0.97; 95% BI: 0.84-1.13 per 5 cm toename) en gewicht in mannen (RR: 1.09; 95% BI: 0.98-1.20 per 5 kg toename) en vrouwen (RR: 1.11; 95% BI: 1.01-1.23 per 5 kg toename) verhoogden het risico van nierkanker. Ook een toename in body mass index sinds 20-jarige leeftijd verhoogde de kans op nierkanker (RR: 1.06; 95% BI: 1.01-1.10). Energie-inname was niet geassocieerd met nierkanker, terwijl de associatie tussen fysieke activiteit en nierkanker nog onduidelijk blijft. Energie-inname en fysieke activiteit verklaarden het gevonden verband tussen BMI en nierkanker niet.

In hoofdstuk 3 hebben we gevonden dat noch de totale groente en fruitconsumptie, noch de consumptie van groenten en fruit geclassificeerd in botanische subgroepen, noch de consumptie van individuele soorten groenten en fruit geassocieerd waren met een verlaagd risico op nierkanker. Daarbij vonden we geen indicatie voor een modificerend effect door roken, BMI of een hoge bloeddruk in de voorgeschiedenis.

Om de associatie van risicofactoren en het specifieke eindpunt "de aanwezigheid van *von Hippel-Lindau (VHL)* gen mutaties" te kunnen onderzoeken, hebben we paraffine-ingebed tumormateriaal verzameld in 51 pathologielaboratoria. Dit is beschreven in hoofdstuk 4. Hiervoor hadden we toestemming van de medisch ethische commissies van de Universiteit Maastricht, PALGA en de Nederlandse Kankerregistratie verkregen. We waren in staat de locatie van het paraffine materiaal te achterhalen voor 273 van de 337 cases. Van 251 patiënten konden we ook daadwerkelijk paraffineblokjes verzamelen. Na revisie door één patholoog, bleek dat het tumormateriaal van 235 cases beschikbaar was voor verdere analyse.

Gebruik makend van PCR-SSCP gevolgd door directe sequencing, vonden we in 61% van de heldercellige niertumoren *VHL* gen mutaties; de meeste mutaties waren truncerende mutaties. We vonden geen verschillen in nucleaire graad, TNM verdeling of stadium tussen tumoren met of zonder *VHL* gen mutaties. Tumoren met *VHL* gen mutaties waren gemiddeld 72.7 mm groot, terwijl de gemiddelde tumorgrootte van

wildtype *VHL* tumoren 65.3 mm was. Dit verschil in grootte was niet statistisch significant (hoofdstuk 4).
Roken is een bekende risicofactor voor nierkanker. Het is aangetoond dat metabolieten uit sigarettenrook mutaties veroorzaken in humaan DNA en dat dit niet alleen plaats vindt in direct blootgestelde weefsels. In hoofdstuk 5 hebben we de associatie tussen roken en nierkanker en *VHL* gen mutaties in heldercellige niertumoren onderzocht. RRs voor totaal nierkanker voor mannen waren 1.52 (95% BI: 0.89-2.59) en 2.07 (95% BI: 1.20-3.56) voor ex- en huidige rokers, respectievelijk, in vergelijking tot nooit rokers. De schattingen voor vrouwen bedroegen respectievelijk 0.95 (95% BI: 0.57-1.59) en 1.37 (95% BI: 0.87-2.16). Voor mannen waren de RRs voor huidige rokers in vergelijking tot nooit rokers respectievelijk 2.34 (95% BI: 0.79-6.94) en 2.95 (95% BI: 0.65-13.28) voor *VHL* gen gemuteerde en *VHL* wildtype tumoren. Voor vrouwen waren deze schattingen respectievelijk 0.82 (95% BI: 0.35-1.93) en 2.04 (95% BI: 0.94-4.45). Roken was geassocieerd met nierkanker bij mannen, maar niet specifiek met *VHL* gen mutaties noch bij mannen, noch bij vrouwen. Dit suggereert dat roken geassocieerd zou kunnen zijn met nierkanker, maar dat dit onafhankelijk van *VHL* gen mutaties verloopt.
Hoge bloeddruk in de voorgeschiedenis en het gebruik van medicatie tegen een hoge bloeddruk zijn gerelateerd aan de kans op nierkanker, maar het is nog onduidelijk welke van deze twee factoren het verhoogde risico veroorzaakt. Een hoge bloeddruk (RR: 1.22; 95% BI: 0.94-1.58) en het gebruik van medicatie tegen een hoge bloeddruk (RR: 1.14; 95% BI: 0.85-1.52) waren geassocieerd met een verhoogd risico op het ontwikkelen van nierkanker, maar dit was statistisch niet significant (hoofdstuk 6). Hoge bloeddruk was geassocieerd met een niet statistisch significant verhoogd risico van heldercellige niertumoren met een *VHL* gen mutatie (RR: 1.34; 95% BI: 0.87-2.07), en vertoonde geen relatie met de kans op heldercellige niertumoren zonder *VHL* gen mutaties (RR: 0.88; 95% BI: 0.51-1.53). Diureticagebruik was niet geassocieerd met *VHL* gen gemuteerde heldercellige niertumoren (RR: 0.91; 95% BI: 0.45-1.81), maar wel met heldercellige niertumoren zonder *VHL* gen mutaties (RR: 2.11; 95% BI: 1.16-3.83). Deze resultaten impliceren dat er mogelijk verschillende routes zijn, via welke hoge bloeddruk en diuretica gebruik leiden tot niertumoren, hoewel foutpositieve bevindingen als gevolg van het uitvoeren van multipele testen en kleine aantallen niet uit te sluiten zijn.
De inname van carotenoïden en vitaminen uit voeding was niet geassocieerd met niertumoren (hoofdstuk 7). Het gebruik van vitamine E, AD en multivitamine supplementen was geassocieerd met een niet statistisch significant verhoogd risico op nierkanker. De resultaten waren suggestief voor hogere RRs in het geval van wildtype *VHL* tumoren in het geval van alfacaroteen, betacryptoxanthine, foliumzuur, vitamine C supplement en multivitamine supplement gebruik.
Deze studie bevestigt de positieve associatie van BMI, roken, een hoge bloeddruk in de voorgeschiedenis en het gebruik van medicatie tegen een hoge bloeddruk en nierkanker. We vonden geen associatie van groente en fruit consumptie en

carotenoïden en vitamine inname en nierkanker. We vonden geen overtuigende verschillen in de associatie tussen risicofactoren en tumoren met en zonder *VHL* gen mutaties, ondanks dat *VHL* gen mutaties gezien worden als een vroege gebeurtenis in de ontwikkeling van heldercellige niertumoren. Een mogelijke reden voor deze bevindingen zou kunnen zijn dat deze risicofactoren een rol in de promotiefase en niet in de initiatiefase van de ontwikkeling van niertumoren een rol spelen. Hypermethylering van de *VHL* gen promoter zorgt er ook voor dat het gen niet afgelezen wordt en er dus geen eiwit gevormd wordt; daarom zou dit ook onderzocht moeten worden. Bovendien is het zo dat in slechts 60% van de heldercellige niertumoren *VHL* gen mutaties worden gevonden. Dit is een indicatie dat andere genen of mechanismen een rol moeten spelen in de ontwikkeling van heldercellige nierkanker, zoals ook het geval is in colorectaal tumoren, waarin verschillende tumor suppressor genen uitgeschakeld en een of meer oncogenen aangeschakeld moeten zijn.
In toekomstig onderzoek zouden andere risicofactoren en/of combinaties van risicofactoren gebaseerd op mogelijke mechanismen verder onderzocht moeten worden. Ook zouden we onze resultaten graag bevestigd of ontkracht zien door andere grote studies. Resultaten uit deze studies en onze resultaten zouden dan ook samen genomen kunnen worden voor een zogenaamde gepoolde analyse, wat zou kunnen leiden tot duidelijkere conclusies. Tenslotte, zouden andere genen die betrokken zijn bij het proces van angiogenese (de vorming van nieuwe bloedvaten), genen die een rol spelen in DNA-schadeherstelprocessen en genen die een rol spelen in apoptose (geprogrammeerde celdood) ook overwogen moeten worden voor verder moleculaire-epidemiologisch onderzoek indien er aanwijzingen zijn voor mutaties of promoter hypermethylering in niertumoren.

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About the author

Boukje Annemarie Cornelia van Dijk was born on 30 May 1974 in 's-Hertogenbosch, The Netherlands. She completed secondary school (VWO) at the Jeroen Bosch College in 's-Hertogenbosch in 1995. In 1993, during secondary school, she also obtained a diploma from Dover High School, Dover, Delaware, USA where she studied for one year. She started her studies Biomedical Health Sciences at the Radboud University Nijmegen (formerly Catholic University Nijmegen) in 1995. As part of her training, she fulfilled three internships. The first was carried out at the laboratory of the department of Pathology, Radboud University Nijmegen Medical Centre, in which an alternative formation pathway of the protein angiostatin in colon carcinoma cancer cell lines was investigated. The second internship concerned a survey on knowledges, attitudes and perceptions of water, diarrhea and hygienic practices and was performed at The Medical Entomology Research and Training Unit/Guatemala, a CDC field office located at the Universidad de Valle in Guatemala. She fulfilled her graduation project at the departments of Epidemiology and Urology, Radboud University Nijmegen Medical Centre, where she carried out a case-control study on alcohol intake, alcohol dehydrogenase and the risk of bladder cancer, which resulted in a publication. After obtaining her MSc degree in Biomedical Health Sciences in 2000, she started her PhD project at the department of Epidemiology, Maastricht University. This project was performed in cooperation with the departments of Urology and Pathology, Radboud University Nijmegen Medical Centre. Her tasks included the collection of paraffin material from kidney (and prostate) cancer cases from pathology laboratories throughout the country, analyzing obtained data and presenting and publishing the results of these investigations. These results have been described in the present thesis. Since June 2005, she has been working as a researcher, investigating the subject of ovarian cancer at the department of Epidemiology, Maastricht University. She will start her new job as a postdoctoral fellow at the department of Clinical Chemistry, Radboud University Nijmegen Medical Centre in February 2006, and will be sent on secondment on a part-time basis to the department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre.